Incorporation of monoclonal antibodies in living rat pheochromocytoma PC12 cells

Evidence for the intracellular formation of immune complex between the incorporated antibody and a target protein

N.A. Alexandrova[a], P.G. Sveshnikov[c], N.K. Nagradova[b], I.D. Grozdova[a,*]

[a] *Polymer Department, Chemical Faculty, Moscow State University, Leninskiy Gori, 119899 Moscow, Russia*
[b] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia*
[c] *Research Center of Molecular Diagnostics and Therapy, Moscow, Russia*

Received 26 June 1998

Abstract PC12 cells permeabilized with a low concentration of digitonin (5 μM) under controlled conditions were loaded with monoclonal antibodies (MoAb) against the regulatory subunit type II (RII) of cAMP-dependent protein kinase. After digitonin removal from the nutrient medium (DMEM) the loaded cells repaired within 20–30 min and recontinued growth. The inserted MoAb stayed in the repaired cells at least for several hours. MoAb inhibiting the cAMP binding activity of neural RII [Grozdova et al. (1992) Biochem. Int. 27, 811–822; Sveshnikova et al. (1996) Biochem. Int. 39, 1063–1070] were shown to bind the target antigen inside the cells and influence the properties of intracellular protein kinases.

Key words: PC12 cell permeabilization; Digitonin; Antibody insertion; A-kinase regulatory subunit type II

1. Introduction

One approach to investigate intracellular regulatory processes is to include specific agents in the cytosol of living cells. Incorporation of enzyme ligands (substrates, inhibitors or antibodies) was employed in direct studies of enzyme localization and activity in different cellular processes, i.e. exocytosis [3,4], mitochondrial functions [5], nuclear translocation of proteins [6], transformation of surface signals [7,8]. Introduction of normally impermeant molecules in numerous cells requires their permeabilization. However, most permeabilizing procedures make it possible to perform the experiments within 10–20 min after membrane disruption [3,4,9]. Time restriction is due to a gradual decline of cell viability presumably caused by leakage of intracellular proteins [4,9]. This explains why the methods of transient cell permeabilization with subsequent reparation of the plasma membrane [10,11] are of special interest.

Recently we have developed a digitonin-mediated permeabilization technique which allows insertion of proteins as large as immunoglobulins (160 kDa) into rat pheochromocytoma PC12 cells which retain their capacity to repair. Now we applied this method for incorporation of anti-RII MoAb previously shown to inhibit completely the cAMP binding activity of neural RII in vitro [1,2]. The present experiments were undertaken to ascertain that the incorporated MoAb form an immune complex with the target antigen inside the cells and to investigate the influence of the inserted MoAb on the activity of intracellular protein kinases.

2. Materials and methods

2.1. Materials

MoAb clone 6 raised against bovine brain RII [1] and mouse normal IgG were purified by chromatography on protein G-Sepharose. Home-made goat anti-mouse IgG antibodies were purified in the same way and conjugated with horseradish peroxidase [12]. Sheep anti-mouse IgG1 antibodies were from Serotec; digitonin, Triton X-100 and Tween 20 from Serva; polylysine and phenylmethylsulfonyl fluoride from Sigma.

2.2. Cell culture

Rat pheochromocytoma PC12 cells were cultured in DMEM (Sigma) supplemented with 7.5% horse serum (Sigma), 7.5% fetal calf serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37°C in 5% CO_2 humidified atmosphere.

2.3. Cell permeabilization and loading with MoAb

This has been described elsewhere [13]. In brief, PC12 cells were seeded in 6-well Costar plates precoated with polylysine (10 μg/ml, 2 ml/well, 2 h, 37°C) at a density of 2×10^6 cells/well. The next day the attached cells were rinsed once with Ca^{2+}-free buffer (140 mM KCl, 20 mM PIPES, 5 μM EGTA, 2 mM $MgCl_2$, 0.1% BSA, pH 7.2) and permeabilized with 5 μM digitonin in the same buffer within 6–8 min (37°C, 5% CO_2) under visual control (phase-contrast microscopy, Nikon, ×400). After digitonin removal 3 μM MoAb, unless otherwise mentioned, was added to the cells in the Ca^{2+}-free buffer (0.5 ml/well) and the plate was either shaken for 15 min at 37°C or pulse-sonicated 4 times for 2 s with 20 s intervals at 37°C in an ultrasonic water bath Sonorex Super RK 510 H. The cells repaired in serum-free DMEM containing 0.1% BSA and 3 μM MoAb within 25–30 min (37°C, 5% CO_2). The cells of each well were harvested, freed of excess protein by centrifugation (three washes with 1 ml PBS) and assayed for protein kinase activity and MoAb presence.

2.4. Assay of cAMP-dependent protein kinase activity in cell extracts

Cells were suspended in 50 μl of 5 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 3 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, disrupted by sonication (3×10 s, 0°C) and centrifuged (10 000×*g*, 10 min, 4°C). The supernatant was assayed for protein kinase activity using histone H1 as substrate [1].

2.5. Quantitative determination of MoAb

Cells were dissolved in 120 μl 1% Triton X-100 in PBS containing

*Corresponding author. Fax: (7) (095) 939-01-74.
E-mail: melik-nubarov@usa.net

Abbreviations: IgG, immunoglobulin G; MoAb, monoclonal antibodies; RII, the regulatory subunit type II of cAMP-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline

PII: S0014-5793(98)00861-8

2 mg/ml BSA. The amount of MoAb in a cell lysate was determined by ELISA using two uncompetitive types of anti-mouse IgG antibodies. Sheep anti-mouse IgG1 antibodies, 2 μg/ml in PBS, were sorbed on a 96-well plate (0.1 ml/well, 2 h, 37°C) and incubated with 100 μl of cell lysate (1 h, 37°C). MoAb bound to sheep antibodies were detected with goat antibodies conjugated to horseradish peroxidase (1 h, 37°C). The enzymatic reaction was developed with *o*-phenylenediamine, 0.8 mg/ml, in 0.1 M citric acid, pH 5.0, 8 mM H_2O_2, and stopped with 50 μl of 25% H_2SO_4. The absorption was measured at 492 nm. MoAb amount in lysates was estimated using a calibration curve (3–100 ng/ml MoAb) and calculated per 10^6 cells.

2.6. Protein determination

Protein was determined in cell lysates by Coomassie staining [14]. MoAb and IgG concentration was measured at 280 nm assuming $\varepsilon_{280}^{0.1\%} = 1.4$.

3. Results and discussion

3.1. Cell permeabilization and reconstruction

In the previous paper we established that permeabilization was accompanied by changes in cell morphology: the intact and repaired cells were round-shaped while the perforated cells looked flat under phase-contrast microscopy. Correlation of these visible changes with cell perforation was checked by trypan blue exclusion and incorporation of low-molecular substances, [^{3}H]inositol and carboxyfluorescein, in digitonin-treated cells [13]. In the present paper the process of cell permeabilization was routinely controlled under phase-contrast microscopy.

Treatment of PC12 cells with various concentrations of digitonin showed that the time required for perforation of all cells in monolayer was inversely proportional to the digitonin concentration in the range of 2–5 μM (Fig. 1). 1 μM digitonin did not perforate the cells within 1 h 20 min. More than 5 μM digitonin affected the cells' capacity to repair.

Cell repair occurred within 20–30 min in the serum-free nutrient medium (DMEM). The amount of repaired cells depended on the conditions of permeabilization, i.e. digitonin concentration and duration of treatment. Digitonin ≥12 μM damaged all cells irreversibly. treatment with 6 μM digitonin during 6 min provided the subsequent reconstruction of 20% of cells. A further decrease in digitonin concentration to 4.8–5 μM drastically increased the amount of repaired cells to 68–75%. Such yield was obtained under optimal conditions for MoAb insertion. Recovery of $>80\%$ of cells correlated with incorporation of a small amount of MoAb, both effects being presumably caused by insufficient digitonin treatment. Cell permeabilization was carried out during the least time necessary for perforation of all cells in monolayer. Longer treatment decreased cell recovery. The exact permeabilization time depended on cell density and was precisely controlled in each experiment. All other steps of the procedure were not accompanied by a reliable cell loss.

Viability of reconstructed cells was testified by their proliferation. The day after permeabilization and reconstruction the cell amount increased 1.24-fold relative to their quantity just after repair and 1.93-fold on the second day. The corresponding values for digitonin-untreated cells were 1.23 and 1.73, respectively. So the repaired cells recontinued growth after the experiment and had the same doubling time, about 30 h, as the untreated cells.

3.2. Insertion of MoAb

This could be achieved after cell treatment with 2 μM digitonin. We used 4.8–5 μM digitonin in the experiments described below. After its removal the perforated cells were incubated with MoAb in Ca^{2+}-free buffer which prevented membrane reparation. Incorporation of similar MoAb quantities was observed irrespective of whether the cells were incubated at this step for 15 min with shaking or pulse-sonicated within 1 min (see Section 2). This indicates that pulse-sonication significantly accelerated MoAb penetration and thus shortened cell residence in the perforated state. To potentiate MoAb intracellular accumulation they were added also in the nutrient medium during cell repair. Evidence for restoration of the cell membrane after digitonin removal was obtained in our previous work [13].

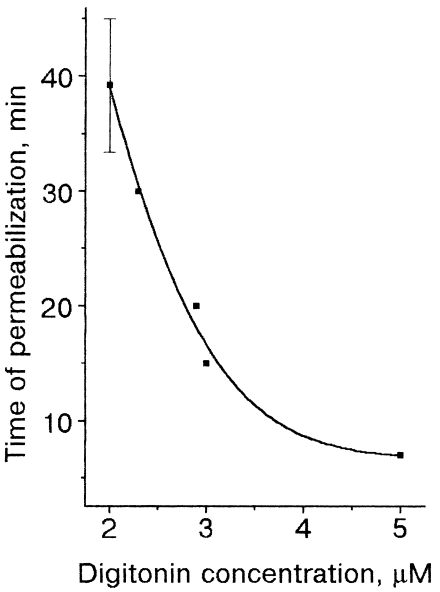

Fig. 1. The relationship between digitonin concentration and the time required for PC12 cells permeabilization. Ordinate: the least time necessary for perforation of all cells in monolayer.

The excess of antibodies was then removed by thorough washing and centrifugation. This procedure also removed dead and irreversibly damaged cells from the cell culture as judged by trypan blue exclusion. The resulting cell cultures contained 95% living cells.

The amount of MoAb included in living cells was determined by ELISA in lysates of the repaired cells. Analysis of cells treated with different concentrations of MoAb showed that the amount of accumulated MoAb directly depended on their concentration used for loading (Fig. 2). When normal serum IgG of non-immune mice was used instead of MoAb, they accumulated inside the cells as well. Similar permeation of immune and non-immune IgG suggests that MoAb incorporation was probably a diffusion-limited process.

Four hours after cell reconstruction the amount of inserted MoAb decreased to 83–85% relative to their content directly after cell loading. In contrast, normal IgG degraded considerably quicker, their amount decreasing to 24–26% within 4 h. The relative stability of MoAB as compared with normal IgG was an indication that MoAb formed an immune complex inside the cells.

3.3. Interaction of MoAb with its antigen inside the cell

It is known that binding of cAMP of the R subunit of

inactive cAMP-dependent protein kinase leads to enzyme activation. MoAb used in the present study were previously shown to inhibit cAMP binding to the neural RII in vitro [1,2]. So abolition of cAMP-induced protein kinase activation in the presence of MoAb can indicate the formation of an immune complex. Keeping this in mind we assayed the ability of protein kinases extracted from the loaded cells to be activated by cAMP. So, the histone kinase activity was measured in the absence and in the presence of cAMP in the cell extracts obtained directly after cell reconstruction (Fig. 3). Control samples underwent the procedure used for loading but were not treated with either digitonin (D), MoAb (B), or both (A). The activities of different samples were expressed in relation to the kinase activity of control (A) tested in the absence of cAMP.

First, we observed that PC12 control cells untreated with digitonin and MoAb (A) had cAMP-sensitive protein kinases, their activity increasing 1.58-fold upon addition of cAMP. Digitonin treatment caused a 22–31% decrease of the kinase activity in the absence and in the presence of cAMP indicating some leakage of the free catalytic subunit and the holoenzyme. However, digitonin did not affect the extent of kinase activation (B).

In contrast, no activation of the histone kinase upon cAMP addition was observed in the cells loaded with MoAb (C). Because cAMP-dependent protein kinase type II is an intracellular enzyme, the inhibitory action of MoAb 6 on its dissociation is conclusive evidence of their penetration inside the cells. The amount of inserted MoAb estimated as described above (see the legend to Fig. 2) accounted for 9.3 ng/10^6 cells which corresponded to 0.1 μM MoAb assuming a PC12 cell diameter equal to 10 μm. This concentration is comparable with 0.5 μM RII endogenous concentration [15] taking into account the leakage of ~1/3 of the enzyme (B). The intracellular concentrations of RII and MoAb were enough for their association (K_d for MoAb 6 and the homologous antigen is equal to 3.8×10^{-9} M [1]). Formation of the immune complex manifested itself in inhibition of cAMP binding. So, the inserted MoAb 6 cross-reacted with RII of PC12 cells and produced remarkably the same effect in vivo as in vitro on isolated bovine, pig and human brain RII [2].

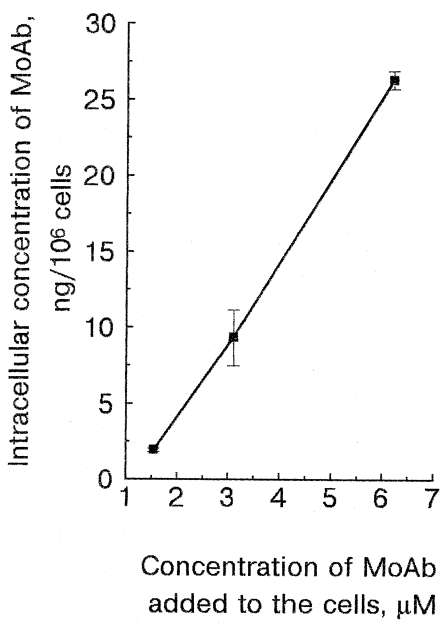

Fig. 2. Dependence of the amount of inserted MoAb on their concentration outside the cells. Cells were permeabilized with 5 μM digitonin in Ca^{2+}-free buffer. Control cells were incubated in the same buffer but without digitonin. Both samples were treated with MoAb at concentrations indicated on the abscissa. The amount of MoAb was determined in cell lysates and calculated per million cells. The difference in MoAb content between the digitonin-treated and control samples is shown on the ordinate.

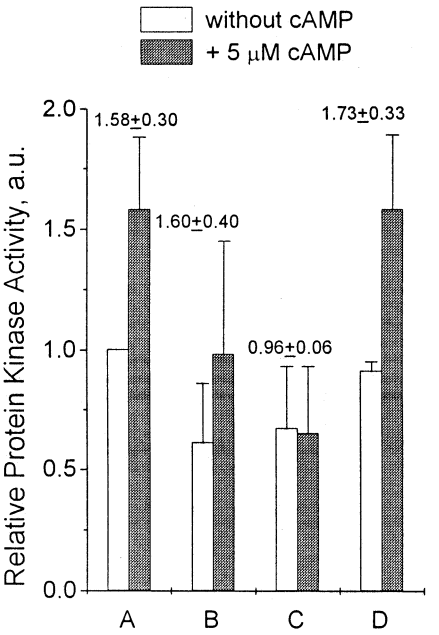

Fig. 3. Histone kinase activity in the extracts of loaded PC12 cells (C) and three control samples (A, B, D) assayed in the absence and in the presence of 5 μM cAMP. PC12 cells were loaded with MoAb according to the procedure described in Section 2. The cells seeded in six wells of a plate underwent the same treatment except for addition into control wells of either digitonin (D), MoAb (B), or both (A). After reconstruction the cells were harvested, similarly treated cells (two wells with loaded cells (C) and two wells of the control B) were combined and washed free of excess MoAb. Each sample was then divided in half. One portion containing $0.5–1\times10^6$ cells was lysed and used for quantitation of MoAb. 3.9 ± 0.6 and 13.2 ± 0.4 ng MoAb/10^6 cell were detected in the control (D) and loaded cells (C), respectively. Cells of the other portion were extracted and the histone kinase activity in the extracts was determined. The activity of each sample is expressed in relation to the activity of the control (A) in the absence of cAMP. The number above the bar shows the extent of kinase activation by cAMP. The results represent mean values ± S.D. obtained in three independent experiments.

Formation of the immune complex was observed only inside the cells but not in the extracts. MoAb that remained outside the unpermeabilized cells due to incomplete washing (3.9 ± 0.6 ng/10^6 cells) did not affect cAMP-induced activation of the extracted protein kinase (Fig. 3D). The result is presumably due to a ~100-fold dilution of RII and MoAb during extraction (0.5–1 μl cell pellet in 50 μl buffer) to concentrations too low for immune complex formation.

In conclusion, the antibodies to the RII subunit of cAMP-dependent protein kinase were inserted into digitonin-permeabilized PC12 cells following a procedure that ensured cell survival after loading. The antibodies formed an immune complex with the target antigen inside the cells and produced the same effect on its properties in vivo as in vitro. Further

studies on remote consequences of MoAb insertion on intracellular protein kinases are in progress.

References

[1] Grozdova, I.D., Kolesnikova, O.O., Koroljova, L.N., Shemchukova, O.B., Sveshnikova, E.V. and Sveshnikov, P.G. (1992) Biochem. Int. 27, 811–822.
[2] Sveshnikova, E.V., Alexandrova, N.A., Grozdova, I.D., Sveshnikov, P.G. and Severin, E.S. (1996) Biochem. Int. 39, 1063–1070.
[3] Ahnert-Higer, G., Bader, M.-F., Bhakdi, S. and Gratzl, M. (1989) J. Neurochem. 52, 1751–1758.
[4] Sarafian, T., Aunis, D. and Bader, M.-F. (1987) J. Biol. Chem. 262, 16671–16676.
[5] Kunz, D., Luley, C., Fritz, S., Bohnensack, R., Winkler, K., Kunz, W.S. and Wallesch, C.W. (1995) Biochem. Mol. Med. 54, 105–111.
[6] Cserpan, I. and Udvardy, A. (1995) J. Cell Sci. 108, 1849–1861.
[7] Rhoads, A.R., Vu, N.D. and Carroll, A.G. (1993) Int. J. Biochem. 25, 79–86.
[8] Zachayus, J.L. and Plas, C. (1993) Mol. Cell. Endocrinol. 92, 15–23.
[9] Esparis-Ogando, A., Zurzolo, C. and Rodriguez-Boulan, E. (1994) Am. J. Physiol. 267, 166–176.
[10] Clarke, M.S.F. and McNeil, P.L. (1992) J. Cell Sci. 102, 533–541.
[11] Hornsby, P.J., Yang, L., Lala, D.S., Cheng, C.Y. and Salmons, B. (1992) BioTechniques 12, 244–251.
[12] Tyssen, P. and Kurstak, E. (1984) Anal. Biochem. 136, 451–457.
[13] Alexandrova, N.A., Posypanova, G.A., Grozdova, I.D., Sveshnikov, P.G. and Severin, E.S. (1998) J. Biochem. Med. Pharmacol. Chem. (Russ.) 2, 16–20.
[14] Bradford, M.M. (1976) Anal. Biochem. 76, 248–254.
[15] Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Proc. Natl. Acad. Sci. USA 71, 3580–3583.