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Bay K8644 like activity of an antibody against a 60 kDa tubular membrane protein

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Partial purification of the dihydropyridine receptor from rat skeletal muscle demonstrated mainly a 60 kDa band in SDS-polyacrylamide gel. An antibody raised against that protein behaved as a calcium channel agonist viz. Bay K8644. The affinity purified antibody, when added to cultured heart cells, increased the beat rate 40–80% depending on the titer of the antiserum. The antibody also woke up the beats of the cells previously blocked with the channel antagonist, nifedipine. Immunoblot analysis indicated that the receptor of this antibody in heart cell membrane is also a 60 kDa protein.

Introduction

Excitation-contraction coupling in muscle is strongly calcium-dependent [1]. A complex infolding of the muscle plasma membrane, the T-tubulin system, contains high concentrations of voltage dependent calcium channels and some cardiac muscle cells have a large calcium-dependent component on their action potential [1]. There are three types of voltage-sensitive calcium channels which are kinetically different and inhibited by different antagonists [2]. The T-type channel has a short open time and is sensitive to high concentrations of cadmium, the L-type channel remains open much longer and sensitive to low concentrations of cadmium, to a snail toxin (CgTx) and are blocked by dihydropyridines (DPH). The N-type channel is sensitive to CgTx and cadmium, but insensitive to DHP.

L-type channels are most important in muscle and can be purified by assaying ^{3}H-dihydropyridine binding. Most groups reported three proteins (140, 60 and 33 kDa, respectively) in their purified preparations, but some reported only two and some four [3–6]. The subunits of 140 kDa and 33 kDa are well characterized as a part of dihydropyridine (DHP) receptor but the contribution of the 60 kDa subunit in the function of calcium channel is controversial. This protein copurifies with DHP receptor and only be separated from 140 kDa and 33 kDa proteins by gelfiltration in presence of urea [3]. Most of the groups have focused their attention towards the 140 kDa subunit and raised antibodies against the subunit [7–9], allowing the cloning of the gene for this protein [10]. However, it is dubious that the 60 kDa protein is a subunit of the skeletal muscle channel. Probably due to this reason people became least interested in the 60 kDa tubular membrane protein. Since our procedure for isolation of DHP receptor from rat muscle gave us a preparation which contained mostly (> 95%) the 60 kDa protein, we have consequently attempted to understand its importance in calcium channel dependent excitation-contraction coupling of muscle cells. In the present report we demonstrated that an antibody raised against the 60 kDa protein functioned as a calcium channel agonist (resemble to Bay K8644) thereby demonstrating the probable importance of this 60 kDa tubular membrane protein in calcium channel functions and its possible clinical significance for the cardiologist.

Materials and Methods

Purification of DHP receptor from rat skeletal muscle was carried out according to the procedure that was described for the isolation from rabbit skeletal muscle [3]. In each step of the isolation procedure the solutions contained a proteinase inhibitor cocktail (iodoacet-

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amide 1 mM, PMSF 0.1 mM and pepstatin 1 μM) to avoid proteolysis. The purification was done upto the WGA-affinity step. Briefly, 400 g (wet wt.) of rat skeletal muscle was homogenized in blender in ice cold buffer (20 mM Mops, 0.3 M sucrose, pH 7.5). Debris, nuclei and crude membranes were removed by low speed centrifugation ($3600 \times g$) for 10 min followed by a centrifugation at the same speed for 40 min. From the supernatant, after addition of KCl 0.5 M final concentration), microsomal fraction was pelleted down by centrifugation at $95000 \times g$ for 30 min. The pellet was washed in 20 mM Mops, pH 7.5, by centrifugation at $95000 \times g$ for 30 min.

The microsomal fraction so obtained was solubilized in buffer containing 4% CHAPS, 7.5% glycerol, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, centrifuged at 160000 $\times g$ for 30 min and the supernatant was incubated with 1 nM ^{3}H-PN 200-110 to trace the DHP receptor during further purification steps. The solubilized microsomal fraction was adsorbed in DEAE-trisacryl (LKB Laboratory) and eluted with buffer (0.3% CHAPS, 0.03% CHS, 0.03% SPC, 5% glycerol, 100 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5) after washing with buffer which contained 75 mM KSCN, 0.1% CHAPS, 50 mM KCl, 0.02% CHS, 5% glycerol. 100 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5. The DHP containing fractions (as judged by radioactivity counts of aliquots from each fraction) were pooled and adsorbed with WGA-affigel 10 (Pharmacia) overnight at 4°C. The DHP receptor was eluted (after washing with buffer containing 1% CHAPS, 0.2% SPC, 0.5 M KCl, 5% glycerol, 1 mM $CaCl_2$, 140 mM NaCl, 20 mM Tris-HCl, pH 7.5) with buffer (0.1% CHAPS, 0.02% SPC, 5% glycerol, 1 mM $CaCl_2$, 140 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.2 M *N*-acetyl-D-glucosamine. The radioactive fractions were collected and pooled for further use.

For antibody production we electroeluted the 60 kDa protein from the preparative gel. The protein was injected into Balb/c mice intraperitoneally – 100 μg of protein emulsified by Freund's complete adjuvant and $5 \cdot 10^7$ pertussis vaccine was used per mouse per injection. After three injections (at one week intervals) blood was taken and serum was assayed for antibody by ELISA using CHAPS solubilized microsomal fraction as antigen. Serum, where pertussis vaccine was used only as antigen was tested side by side as control.

The antigen specific immunoglobulin fraction was purified by affinity chromatography using Affigel 10 according to Pharmacia. Purified 60 kDa protein was coupled with CNBr-activated Affigel 10. The specific antibody was absorbed with the affinity media, washed thoroughly with 0.5 M NaCl and eluted with glycine-HCl buffer, pH 3.5. The antibody so obtained was neutralized with bicarbonate buffer and dialysed against PBS. Immunoglobulin obtained from 1 ml of serum was concentrated in speed vac and finally adjusted to 0.3 ml.

In order to detect the effect of the antibody on calcium channels we have used beating heart cells in monolayer culture. Neonatal heart cells were dissociated with pancreatin from freshly dissociated hearts of newborn rats and plated into Linbro 24 well plate. At the density of $5 \cdot 10^5$ cells/ml, the cells form a monolayer of fibroblast, myocytes and a few macrophages [11]. After 24 h of plating, the myocytes became electrically connected and beat together as a network. The beat rate was counted using an inverted phase contrast microscope. The rate varied from 70 to 100 per min from day to day but was quite from well to well at a given day. after 3 days we have added those agents (preincubated at 37°C for 10 min) and measured the changes in the beats of the heart cells in culture using a phase contrast microscope ($\times 20$ magnification) in an environment of 37°C, 5% CO_2/95% air.

For immunoblot analysis the purified WGA-fraction, microsomal fractions from rat skeletal muscle and heart were ran in 10% SDS-polyacrylamide gel and transblot into 'Immobilin P' membrane (Millipore) according to Towbin et al. [12]. The antibody binding was visualized by avedin-biotin technique using 'ABC-Kit' of Vector Laboratory. Electrophoresis was carried out according to Laemmli [13] and protein was determined by Lowry et al. [14].

Results and Discussion

The SDS-polyacrylamide gel electrophoresis revealed, after double silver staining, that the purified DHP-receptor from rat skeletal muscle contained predominantly the 60 kDa protein – no significant contribution of the other subunits of calcium channel was observed in this preparation. When the protein was charged in high amount, the 140 kDa as well as 33 kDa bands could be visualized (Fig. 1). The DHP binding specificity of this preparation was found to be comparable to those reported for rabbit muscle [3] but there was large differences in the protein contents of the two preparations. Fig. 2 shows the Schart plot of the (+)^{3}H-PN 200-110 binding of the purified WGA-fraction from rat skeletal muscle. Hence the isolation procedure yielded a virtually pure population of the 60 kDa tubular membrane protein in which the 140 kDa and 33 kDa DHP receptor subunits present as a minor contamination.

In order to study the potential role of this 60 kDa protein in the function of calcium channels, and since there are several reports where antibodies against crude T-tubulin membranes interfere with calcium channels [8,9], we raised an antibody against this 60 kDa protein and tested its effect on the excitation-contraction coupling.

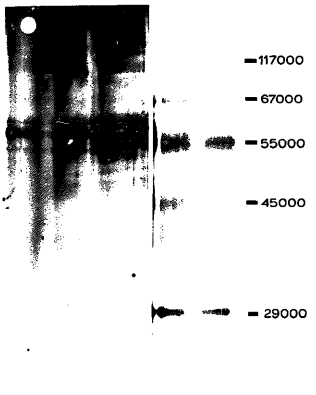

Fig. 1. SDS-polyacrylamide gel electrophoresis (10%) of purified calcium channel receptor from rat skeletal muscle. The gel was double stained with silver reagent. Lanes A, B and C are the purified WGA-fraction – 5, 15 and 30 μg of protein were loaded, respectively.

The mice immunized with 60 kDa protein gave titers against crude receptor fraction (CHAPS solubilized microsomal fraction) which ranged from 1:30000 to 1:60000. Titers were determined by ELISA technique using 'ABC-Kit' of Vector Laboratory.

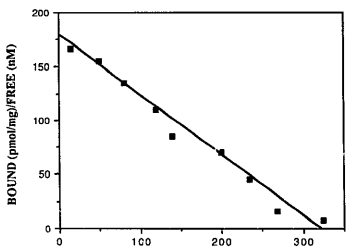

Fig. 2. $(+)^3$H-PN 200-110 binding capacity of purified DHP receptor from rat skeletal muscle. The protein was incubated (final volume 0.1 ml) with increasing concentrations of radiolabelled PN 200-110 for 1 h at 4°C. The bound and unbound ligands were separated by filtration through 1.8 ml Sephadex G-50 columns. Nonspecific binding was measured in the presence of 1 μM of unlabelled (+) PN 200-110.

Effects of the antibody and other chemical agents on the beating heart cells in culture was shown in Fig. 3. Cobalt, which blocks the N-type calcium channels but not DHP receptors, stopped completely the beating at the dose of 1–10 mM (final concentration). On the other hand, TTX only slowed down the rate slightly at the concentration of 10^{-5} M. Noradrenaline, which is not related to calcium channels, did not affect the beat rate even at the final concentration of 1 mM but increased the force of contraction. Nifedipine, a dihydropyridine calcium channel blocker, also stopped the beating within 10 min at a final concentration of 20 nM and the cells remained blocked for at least 48 h. In contrast, Bay K8644, a dihydropyridine agonist (at 10 nM final concentrations), increased the beat rate to about 2-fold of normal value within as soon as 5 min.

In the second set of experiments, we have studied the competitive effect of nefidipine and Bay K8644 on the heart cell beating. The results (Fig. 4) showed that the heart cells when treated with nifedipine stopped beating but could be awakened by Bay K8644 at 10 nM concentration within 1 h. In contrast, noradrenaline did not wake up the nifedipine blocked cells even at 1 mM concentration.

Addition of the anti 60 kDa antibody (both crude and affinity purified) in the heart cell culture had effects identical to Bay K8644 (Fig. 3). This antibody (preincubated at 37°C for 10 min) increased the beat rate by 40–90% over control serum (or immunoglobulins from control serum) depending on the titer. The effect was rapid (within 15 min) and long lasting (about 1–2 h). The second effect, viz. the awakening of nifedipine-blocked cells, had a slower onset (Fig. 4) – just like Bay K8644. Cells treated with nifedipine (20 mM) stopped beating but could be awakened by the addition of the anti 60 kDa serum or affinity purified antibody (10–20 μl/200 μl culture medium). The time required for awakening varied from serum to serum depending on the tier, but was always between 1 and 2 h. The rate was recovered rapidly afterwards and became almost normal within 36 h. Control serum (or immunoglobulins from control serum) had no effect on the beating of the nifedipine-blocked heart cells in culture (Fig. 3 and Fig. 4).

In order to detect the binding site of the antibody in the heart cells we have performed immunoblots of the proteins of the WGA-fraction from rat skeletal muscle, microsomal fraction of rat skeletal muscle and heart cells. In all the three cases the antibody recognized a single band near the 60 kDa region (Fig. 5).

The results described above demonstrated that the 60 kDa protein, which always co-purifies with the 140 kDa dihydroptridine receptor protein, has a role in excitation-contraction coupling of heart muscle cells – though

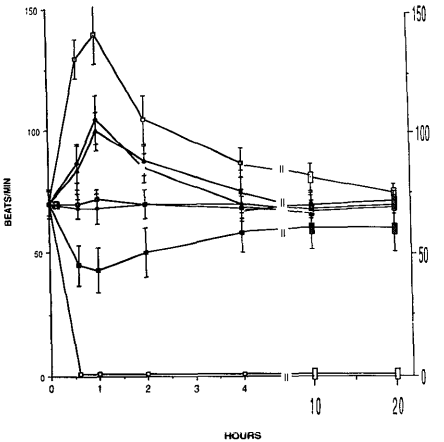

Fig. 3. Effects of different chemical agents and the anti 60 kDa antibody on the beats of heat cells in culture. Cells were grown in 24 well plates containing 200 μl MEM per well and the chemicals/antibody were added to the medium to study the change in beat rate. □, cobalt and nifedipine; ■, TTX; ▲, antiserum; ◆, affinity-purified antibody; ⊡, Bay K8644; ◙, noradrenaline and ❖, normal serum. (a) indicates the point of addition of different agents.

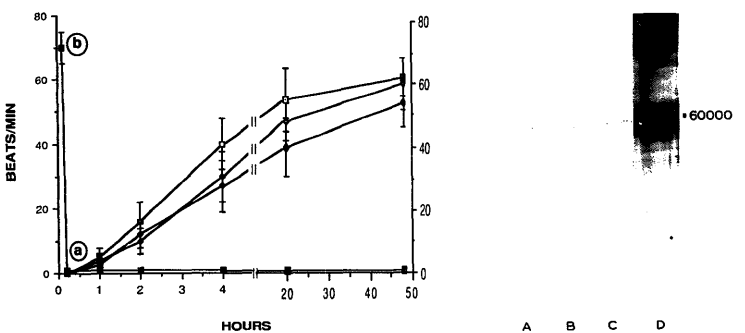

Fig. 4. Competition of Bay K8644, noradrenaline, anti 60 kDa antibody and control serum with nifedipine in modulating the beat rate of heart cells in culture. □, Bay K8644; ■, control serum and noradrenaline; (◇), anti 60 kDa serum and ◆, affinity-purified antibody. (b) point of addition of nifedipine. (a) point of addition of agents interfering with nifedipine.

Fig. 5. Identification of membrane antigen of rat skeletal and heart muscles recognized by anti 60 kDa serum. Lanes A, B and C are the immunoblots of the WGA-fraction, total rat skeletal muscle membrane and total rat heart muscle membrane, respectively; Lane D is the total protein stain of the WGA-fraction.

it was shown not to bind to the dihydropyridines as judged by the photo-affinity labelling of the proteins in polyacrylamide gel [15]. In our preparation about 95% of the protein was found to be 60 kDa subunit. The function of this protein is presumably related directly or indirectly to the calcium channel. It is clear that the action of the anti 60 kDa protein was not mediated through adrenergic receptor as its action was not like that of noradrenaline on the cultured heart cells. On the other hand, the interaction of the antiserum with the calcium channel was indicated from the similarities between the actions of the antiserum and that of Bay K8644, the calcium channel agonist. In heart cells the antibody mediated its action through binding to 60 kDa protein – this was evident from immunoblot analysis. The existence of the 60 kDa tubular membrane protein in heart cell was already well documented [16].

The understanding of biochemical properties of calcium channel became great importance as they control the cardiovascular system. For studying calcium channel function now-a-days the major tools are the synthetic drugs which interferes with the calcium channels, viz. dihydropyridines. It is interesting that one optical enantiomer of DHP, Bay K8644, functions as an agonist of the calcium channel. For biochemical purpose these DHPs can be used as a good probe for the calcium channel, but they are weak in action when interact with functioning heart cells [1]. Antibodies which act either as an agonist or as an antagonist of the calcium channel functions could solve the problem – strong antibodies may help the whole cell physiologist as well as the cell biologist to study the calcium channel functions in muscle cells. From a clinical point of view, our study raised the question that whether the patients who are suffering from abnormal heart beats are the victims of an autoantibody against the calcium channel. Testing of patient's serum on cultured heart cells may resolve the question, within very short period and by a simple technique.

The knowledge of physiology, biochemistry, molecular biology and whole cell physiology of the calcium channel is advancing rapidly. The immunological approaches will also be very helpful in understanding the biochemical as well as physiolgoical properties of calcium channels. To our knowledge, this is the first report on the functional significance of 60 kDa tubular membrane protein of skeletal muscle.

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References

1 Miller, R.J. (1987) Science 235, 46–52.
2 Nowycky, M.C., Fox, A.P. and Tsien, R.W. (1985) Nature 316, 440–443.
3 Borsotto, M., Barhanin, J., Fosset, M. and Landusky, M. (1985) J. Biol. Chem. 260, 14255–14263.
4 Curtis, B.M. and Catterall, W.A. (1984) Biochemistry 23, 2113–2117.
5 Leung, A.T., Imagawa, T. and Cambell, K.P. (1987) J. Biol. Chem. 262, 7943–7946.
6 Sharp, A.H., Imagawa, T., Leung, A.T. and Campbell, K.P. (1987) J. Biol. Chem. 262, 12309–12315.
7 Morton, M.E. and Forehner, S.C. (1987) J. Biol. Chem. 262, 11904–11907.
8 Schmid, A., Barhanin, J., Mourre, C., Coppola, R., Borsotto, M. and Landuski, M. (1986) Biochemistry 25, 3492–3495.
9 Schmid, A., Barhanin, J., Mourre, C., Coppola, R., Borsotto, M. and Landuski, M. (1986) Biochem. Biophys. Res. Commun. 139, 996–1002.
10 Tanabe, T. (1987) Nature 328, 313–318.
11 Burt, J.M. (1982) J. Mol. Cell. Cardiol. 14, 99–110.
12 Towbin, H., Stachlin, T. and Gordon. J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
13 Laemmli, U.K. (1970) Nature 227, 680–685.
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 143, 265–270.
15 Glossman, H., Ferry, D.R., Striessning, G.A. and Moosburger, K. (1987) Trends Pharmacol. Sci. 8, 95–100.