On the mechanism of $\beta$-adrenergic regulation of the Ca channel in the guinea-pig heart*

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Abstract. Dose-response relations for the increase in the amplitude of Ca current ($I_{Ca}$) on external application of isoprenaline (ISP) and internally applied cyclic AMP (cAMP) or catalytic subunit of cAMP-dependent protein kinase (C subunit) were established in single ventricular cells of the guinea pig. An intracellular dialysis technique was used. The threshold concentration was for ISP $10^{-9}$ M, for cAMP $3 \mu$M (pipette concentration to which $10^{-5}$ M 3-isobutyl-1-methylxanthine was added) and for C subunit around 0.4 $\mu$M (pipette concentration). The concentrations for the half-maximal effect were $3.7 \times 10^{-8}$ M (ISP), 5.0 $\mu$M (cAMP) and 0.95 $\mu$M (C subunit) and for the maximum around 0.4 $\mu$M (pipette concentration). The effects of cAMP and C subunit on $I_{Ca}$ were non-additive to those of ISP. From these data the relationship both between concentrations of ISP and CAMP and between those of ISP and C subunit in terms of their effects on $I_{Ca}$ could be estimated and were compared with those obtained in broken cell preparations.

A competitive inhibitor of phosphorylation, 5'-adenylylimidodiphosphate (5 mM), greatly reduced the effects of ISP and C subunit on $I_{Ca}$. Cell dialysis with 3 mM adenosine-5'-(7-thio)-triphosphate, which produces a dephosphorylation-resistant phosphorylation, markedly potentiated the effects of ISP and cAMP on $I_{Ca}$.

The results support the hypothesis that phosphorylation of a protein within, or close to, the Ca channel by cAMP-dependent protein kinase is the mechanism of $\beta$-adrenergic stimulation.

Key words: Cardiac myocytes - Ca current - Isoprenaline - Cyclic AMP - cAMP-Dependent protein kinase - Protein phosphorylation

Introduction

$\beta$-Adrenergic stimulation of heart muscle cells results in an increase in $I_{Ca}$. In analogy to the $\beta$-adrenergic regulation of glycogen metabolism, this increase is thought to be mediated by a cascade reaction (for reviews see Tsien 1977; Drummond and Severson 1979; Reuter 1983): a $\beta$-adrenergic agonist activates adenylate cyclase to produce cAMP which in turn dissociates the inactive holoenzyme of cAMP-dependent protein kinase to yield active catalytic subunit (C subunit). Finally, C subunit catalyses the phosphorylation of a protein within, or close to, the Ca channel. This hypothesis is supported by the observation that application of membrane-permeable cAMP analogues or direct intracellular application of cAMP elevates the plateau of the action potential which has been shown to be due to an increased $I_{Ca}$ (reviewed by Tsien 1977; Reuter 1983). Another piece of evidence is that injection of C subunit also increases $I_{Ca}$ (Osterrieder et al. 1982; Brum et al. 1983). The final step of the cascade, i.e. phosphorylation of a Ca channel-related protein, however, remains still controversial with respect to the nature of the protein and its relation to the Ca channel (Rinaldi et al. 1981, 1982; Flockerzi et al. 1983; Horne et al. 1984).

The electrophysiological experiments combined with injection of cAMP or C subunit were up to now qualitative in nature, i.e. the threshold and the maximum concentrations remained unknown. For a final conclusion on the mechanism of $\beta$-adrenergic regulation of $I_{Ca}$, a quantitative study is necessary, which allows comparison of electrophysiological and biochemical data. Especially, the following predictions of the hypothesis should be verified: 1) the increasing effects on $I_{Ca}$ of cAMP and C subunit should occur in their physiological concentration range in a dose-dependent manner; 2) the effects of cAMP and C subunit should saturate at a similar increase in $I_{Ca}$ to that of $\beta$-adrenergic agonist, and should be non-additive; 3) the effects of all these agents should depend on the hydrolysis of ATP.

In the present study, these predictions have been examined in single ventricular myocytes using a cell dialysis technique. The results were consistent with these predictions and hence provide further support for the phosphorylation hypothesis for the regulation of cardiac Ca channels.

Methods

Preparation of single myocytes. Single ventricular myocytes were isolated according to procedures described elsewhere (Isenberg and Klöckner 1980, 1982; Taniguchi et al. 1981) with slight modifications. Guinea-pigs weighing 250 - 500 g were anesthetized with pentobarbital sodium (30 mg/kg, i.p.). Under artificial respiration the chest was opened. The aorta was then cannulated in situ and immediately perfused with Tyrode solution at a pressure of 60 cm H$_2$O, before the heart was dissected out. After wash out of the blood from...
the isolated heart with Tyrode's, about 50 ml of nominally Ca²⁺ Tyrode solution was perfused. Fifty milliliter of low Ca²⁺ Tyrode solution containing 30 mg collagenase (Type 1, Sigma, St. Louis, MO, USA) and about 50 μM Ca²⁺ was then recirculated at a rate of approximately 10 ml/min for 20–30 min. Thereafter, the enzyme was washed out by 50 ml of a high K⁺ and low Cl⁻ solution (KB medium). All solutions were warmed to 37°C and oxygenated. Finally, the cells were stored in the KB medium at room temperature for later electrophysiological experiments.

**Solutions.** The Tyrode solution contained (in mM): NaCl 112, NaHCO₃ 24, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.0, glucose 10, and HEPES 5. The solution was equilibrated with 95% O₂ and 5% CO₂ and the pH was 7.4. Nominally Ca-free Tyrode solution was prepared by simply omitting CaCl₂ from the Tyrode solution. The KB medium contained (in mM) K-glutamate 50, KCl 50, taurine 20, KH₂PO₄ 20, MgSO₄ 3, glucose 10, HEPES 10, and EGTA 0.5; pH was adjusted to 7.4 by adding KOH. The internal solution in the recording pipette contained (in mM) K-aspartate 80, KCl 50, KH₂PO₄ 10, MgSO₄ 1.0, HEPES 5, Na₂ATP 3, EGTA 0.02; pH was adjusted to 7.3–7.4 by KOH. In some experiments with C subunit, K-aspartate and KCl were 131 and 2 mM, respectively. There was no difference in the effects of C subunit in either of the two solutions.

(−)-Isoprenaline (isoproterenol, ISP, 1 mM, Sigma, St. Louis, MO, USA) was dissolved in distilled water containing 1 mM ascorbic acid. Adenosine-3',5'-cyclic monophosphate (cAMP; Serva, Heidelberg, FRG) and 3-isobutyl-1-methylxanthine (IBMX; Sigma) were also dissolved in distilled water as 1 mM stock solutions and were stored below -18°C. 5'-Adenylyl-imidodiphosphate (AMP-PNP; lithium salt, Boehringer, Mannheim, FRG) and adenosine-5'-(7-thio)-triphosphate (ATP3S; lithium salt, Serva, Heidelberg, FRG) were substituted for ATP in the internal solution. 2,4-Dinitrophenol (DNP, Sigma) was directly dissolved in the internal solution.

**Preparation of C subunit.** The catalytic subunit of cAMP-dependent protein kinase II (C subunit) was obtained from bovine heart as previously described (Beavo et al. 1974a; Hofmann 1980). The enzyme was chromatographed on a Biogel-P6 column (2.5 × 9 cm) equilibrated in a solution containing 150 mM K-aspartate, 50 mM EGTA and 2 mM HEPES at pH 7.2. Protein containing fractions were concentrated by several steps of vacuum dialysis at 4°C and denatured protein was removed by centrifugation. The supernatant contained up to 10 mg/ml C subunit and the purity was more than 95%. The enzyme solution (approximately 100 μM) could be stored for more than a week at 4°C for later electrophysiological experiments.

**Electrophysiological experiments.** A tissue piece was dissected from the left ventricle immersed in KB medium and the cells were dispersed in the recording chamber (0.2–0.3 ml volume) filled with Tyrode solution. After the cells had settled on the glass bottom of the chamber, superfusion was started at a rate of 0.5–1 ml/min at 32–35°C.

Heat-polished patch electrodes were prepared from Pyrex glass capillaries according to Hamill et al. (1981). The electrodes had a inside tip diameter of 2–4 μm and a resistance of 2–5 MΩ when filled with the internal solution. After establishing a giga-Ω seal by suction, the membrane patch was disrupted by further suction of -30 to -60 cm H₂O. The same electrode was used both for the measurement of membrane current (single-electrode voltage clamp) and for the cell dialysis. For the current measurement the series resistance due to the electrode resistance was compensated by current chopping method (Isenberg and Klöckner 1980; Iijima et al. 1985) with a frequency of 20–30 kHz. A liquid junction potential between the pipette solution and the Tyrode solution of -9 mV was corrected. The input membrane resistance and capacitance of the cell were measured by applying either a small hyperpolarizing current in the current-clamp condition or a -10 mV step pulse in the voltage-clamp condition. The potential response in the former was fitted to a simple exponential curve, whereas in the latter, capacitive charge transfer and steady-state current level were measured. The values of resistance and capacitance obtained from these two different methods agreed within ±20%.

Action potentials and membrane currents were displayed on a storage oscilloscope (Tektronix 5113) and were stored on magnetic tape (Hewlett Packard 3964) for later computer analysis (Nicolet MED-80).

**Internal dialysis of cells.** The perfusion device for the internal dialysis is similar to those described by Soejima and Noma (1984) and Matsuda and Noma (1984) and has been reported in detail elsewhere (Trautwein and Kameyama 1985). A thin heat-pulled polyethylene tube (tip diameter 10–40 μm) connected with a stainless steel tube was inserted into the glass pipette so that the tip of the plastic tube was as close to the pipette tip as 80–200 μm. A change of solution in the pipette was carried out by applying a negative pressure (-20 to -50 cm H₂O) to the back end of the pipette and, simultaneously, a positive pressure (10–100 kPa) to the inner tube. During this procedure a slight swelling of the cell was often observed on the TV monitor, without any sign of a change in the giga-Ω seal.

**Efficacy of dialysis.** The diffusion of molecules from the pipette into the cell was simulated in a simplified compartment model (cf. Hille 1977). Figure 1 shows concentration distributions along the pipette and the cell 6 min after the start of dialysis with diffusion coefficients (D) of 1.0–7.0 × 10⁻⁶ cm²/s. Assuming spherical shapes of diffused molecules and the temperature to be 35°C, these D values may correspond to apparent molecular weights (MW) of 300, 3,300, 8,000, 34,000 and 85,000, respectively. The time required for the 30th compartment (end of the cell) to reach 90% of the pipette concentration (T₉₀) was inversely related to D, e.g. T₉₀ = 2.4 min with D = 7.0 × 10⁻⁶ cm²/s and T₉₀ = 12 with D = 1.4 × 10⁻⁶. If these assumptions are valid for the diffusion of C subunit (MW = 40,000), the concentration increased by diffusion into the cell can be estimated to reach 50% and 90% of the pipette concentration 4.2 and 13 min after the start of cell-dialysis. In the experiments, however, when Iₑ was increased by C subunit it tended to decrease in 10–20 min (see Results, p. 288) and this might produce an apparent steady-state of Iₑ. Therefore, we tentatively estimated the concentration of C subunit in the cell to be at least half of that in the pipette since the steady-state of Iₑ was reached after 5–15 min. In the case of cAMP, although T₉₀ was estimated as 2.5 min it would be slowed by the phosphodiesterase activity, and/or a steady-state concentration in the cell would be smaller than...