Altern. Animal Test. Experiment. 6 (1),58-64 (1999)

Effects of Ca Channel Blockers under Different Lighting Conditions in the Chick Embryonic Heart.

Hiroyuki Miyazaki, Takashi Sugiyama and Hideyo Shimada

Division of Pathophysiology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108-8641, Japan

Abstract

The pharmacological effects of Ca channel blockers on electrocardiogram (ECG) in the chick embryonicheart were investigated under different lighting conditions. The fertile eggs of White Leghorn chickens were incubated in dark conditions and used on day 16 of incubation. Three different types of Ca channel blocker, nicardipine, diltiazem and verapamil were injected into the air sac of the fertile eggs under light conditions (450 Lux) or dark conditions (12 Lux). The bipolar lead of ECG patterns was recorded using ECG systems. Heart rates (HRs) were calculated from R-R intervals. Under light conditions, 3 kinds of Ca channel blockers caused a decrease in HRs in a dose-dependent manner and also arrhythmia was provoked. These phenomena were observed more clearly under dark conditions. Furthermore, these enhancements of the drug effects under dark conditions were also observed by concomitant injection of 15-50μg/egg of melatonin even under light conditions. In conclusion, these results suggest that chick embryos are very useful models for investigating the chronopharmacological effects and for evaluating drug interaction of cardiovascular drugs.

Keywords: *Ca channel blocker, chick embryo, electrocardiogram, chronopharmacology, melatonin*

Introduction

Chick embryos have been widely used in physiological and toxicological experiments for many years (Karnofsky 1955, Shepard 1976). While the use of mammalian in various experiments has been criticized from an ethical viewpoint in recent years, interest in the use of the chick embryo as an alternative to mammalians has increased. We have already reported that chick embryos were useful for evaluating the pharmacological and toxicologi-

Correspondence: Takashi Sugiyama, Ph.D.
Division of Pathophysiology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108-8641, Japan
Tel / Fax 03-3446-9036
E-mail: sugiyama@platinum.pharm.kitasato-u.ac.jp

cal effects of cardiovascular drugs as an alternative method (Miyazaki et al. 1994, Sugiyama et al. 1997). Furthermore, chick embryos may be used as a technique in the field of chronopharmacology, because the electrocardiogram (ECG) patterns through the incubation and examination periods were strongly influenced by the lighting conditions (Yoshiyama et al. 1995). In this report, we evaluated the effects of different lighting conditions and pineal hormones on ECG patterns in chick embryos using 3 kinds of Ca channel blockers.

Materials and Methods

Eggs and incubation

Fertile eggs of White Leghorns were obtained from Ohmiya Poultry Laboratory (Ohmiya). All eggs were incubated at 37.6 ± 0.2℃ at a relative humidity of about 65.5% under dark conditions and were turned automatically every hour (Showa Incubator Laboratory). Fertile eggs were used on day 16 of incubation.

Drugs used

Nicardipine hydrochloride (Perdipine, Yamanouchi) and verapamil hydrochloride (Vasolan, Eizai) were diluted by physiological saline. Diltiazem hydrochloride (Herbesser, Tanabe) was dissolved in pure water and diluted by physiological saline. A mg of melatonin (Aldrich) was dissolved in 1 mL of DMSO (Wako) and diluted by physiological saline. Forty-five mg of chloralose and 450 mg of urethane were dissolved in 1.0 mL of water and a 0.1 mL of the solution was injected into the air sac of fertile eggs to calm the embryo in the egg shell.

Electrocardiogram (ECG) recording systems for chick embryos

The ECG waves in the chick embryos were recorded using the previously reported methods (Sugiyama 1996). The needle-electrodes were inserted into 2 diagonal holes on the "equator" and 1 hole on the "south pole" of the egg. Two needles were used as a bipolar lead for the embryonic heart and 1 needle was used as a ground lead. These needles were then connected to the electrocardiograph equipment (AVB-21, Nihon Koden). ECGs were recorded as bipolar waves between the needles on a thermal array recorder (PTA-1100, Nihon Koden) and heart rates (HRs) were calculated from R-R intervals.

Effects of lighting conditions

The experiment was performed under light conditions (under the fluorescent lamp, 450 Lux) or dark conditions (under the red safety lamp, 12 Lux) for 2 hours after acclimatization. A cloralose and urethane (CU) solution was injected into the air sac of the fertile eggs on day 16 of incubation. After 20 minutes of CU solution pre-treatment, a single injection of varying doses of drugs was made into the same site of the eggs.

Effects of melatonin

Fifteen to 50μg of melatonin, that were previously found to have no effect on ECG patterns, was concomitantly injected with varying doses of Ca channel blockers.

Statistical analysis

All results are given as mean ± S.E.M. The data of multiple groups were analyzed by Bartlett's test for homogeneity of variance. They were then analyzed by one-way ANOVA when the variance was homogenous, or by Kruskal-Wallis's test when it was not homogenous. If there was a significant difference among the groups, a mean or rank multiple comparison test was conducted by Dunnet's test. The data of the 2 groups were analyzed by F-test followed by Student's t-test or Aspin-Welch's t-test. The fiducial limit of 0.05 or 0.01, two tails, was used as the criterion for significance.

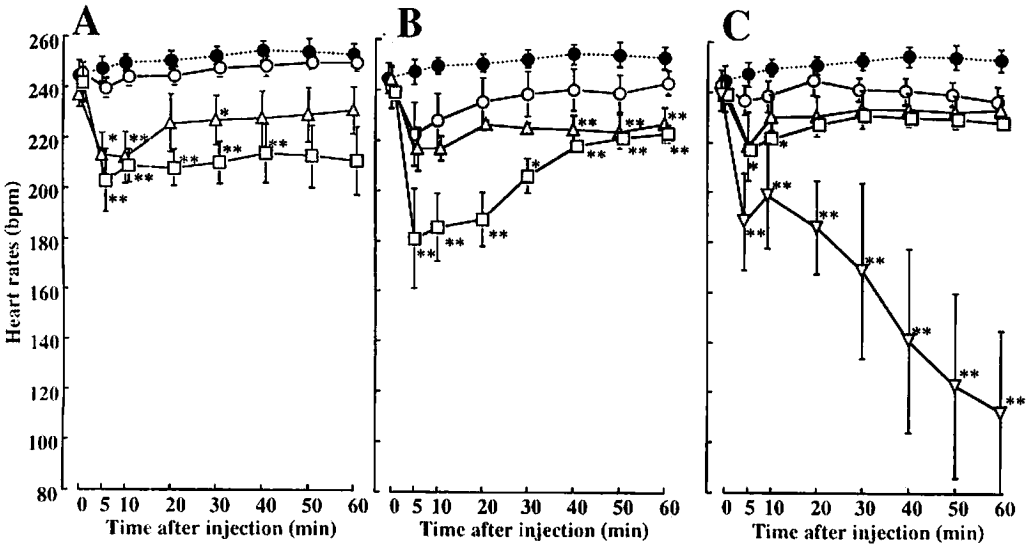

Fig. 1. Heart rate changes in chick embryos treated with Ca Channel blockers under light conditions.
A: 0 μg (●), 10 μg (○), 30 μg (△) and 100 μg (□) of nicardipne was injected into the air sac.
B: 0 μg (●), 30 μg (○), 100 μg (△) and 300 μg (□) of diltiazem was injected into the air sac.
C: 0 μg (●), 3 μg (○), 10 μg (△), 30 μg (□) and 100 μg (▽) of verapamil was injected into the air sac.
Fertile eggs were used on day 16 of incubation. Each value represents the mean±S.E.M. for 5 embryos.
*P<0.05, **P<0.01, significantly different from 0 μg/egg dose group by Dunnett's test.

Results

Effects of Ca channel blockers under the different lighting conditions

Under light conditions, the injection of nicardipine caused a decrease in HRs in a dose-dependent manner and arrhythmia due to A-V block was induced by dosage of 100 μg/egg (Fig.1A). Under dark conditions, these tendencies were more marked, i.e., in the 30μg dose group the results were equivalent to the 100μg dose group in light conditions (Fig.2A, 3A). Further, arrhythmia was followed by severe bradycardia in the 100μg dose group.

Diltiazem caused by the dose-dependent decrease in HRs in both light and dark conditions and induced arrhythmia at all doses (Fig.1B, 2B). While the magnitude of the decrease in the HRs by diltiazem was more apparent in dark conditions as in the case of nicardipine, the decreases were less prominent than those induced by nicardipine (Fig.3B).

In verapamil, no dose-dependent change of the HRs was observed under 30μg. However, prominent bradycardia and arrhythmia were provoked in the 100μg dose group in light conditions (Fig.1C). Under dark conditions, the dosage which induced similar violent changes was 30μg, 1/3 the above dose (Fig.2C, 3C). In the 100μg dose group, arrhythmia and bradycardia were followed by cardiac arrest.

Effects of melatonin

Under light conditions, melatonin alone had no effect on the heart rate or ECG patterns at doses of 50μg and below (data not shown). However, melatonin enhanced the effects of the 3 kinds of Ca channel blockers previously mentioned when they were concomitantly injected with them. This enhancement effect was most prominent when concomitantly injected with nicardipine (Fig.4).

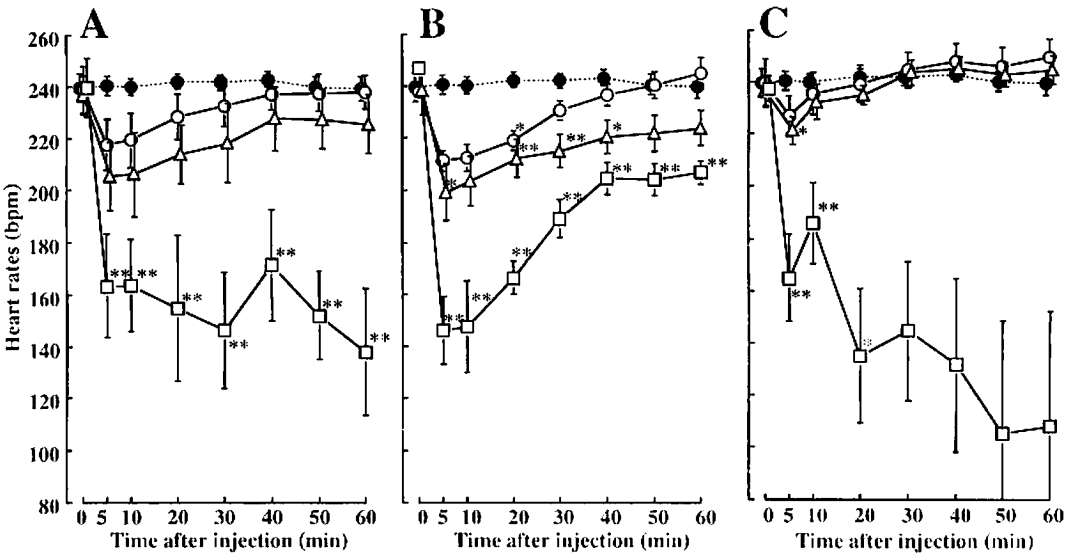

Fig. 2. Heart rate changes in chick embryos treated with Ca Channel blockers under dark conditions.
A: 0 μg (●), 10 μg (○), 30 μg (△) and 100 μg (□) of nicardipne was injected into the air sac.
B: 0 μg (●), 30 μg (○), 100 μg (△) and 300 μg (□) of diltiazem was injected into the air sac.
C: 0 μg (●), 3 μg (○), 10 μg (△) and 30 μg (□) of verapamil was injected into the air sac.
Fertile eggs were used on day 16 of incubation. Each value represents the mean±S.E.M. for 5 embryos.
*P<0.05, **P<0.01, significantly different from 0 μg/egg dose group by Dunnett's test.

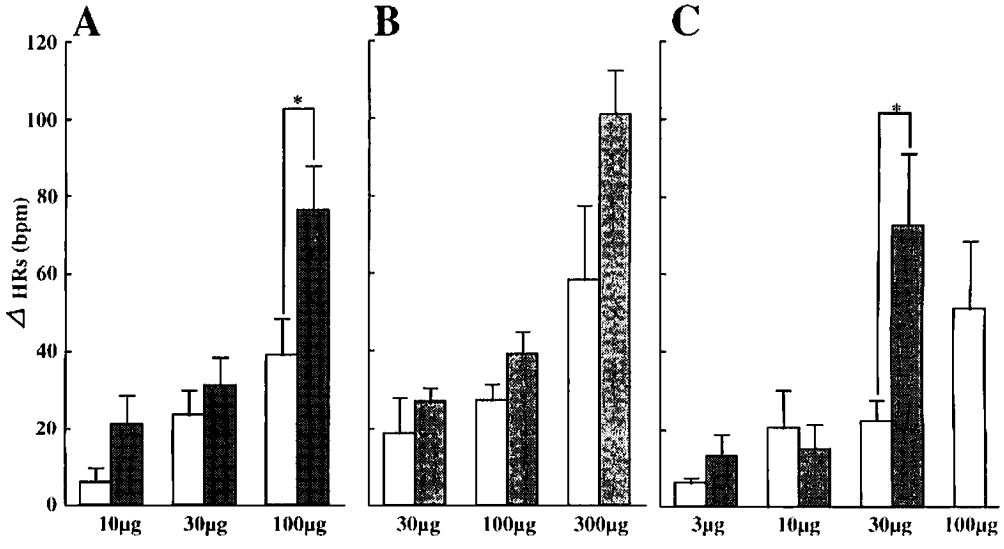

Fig. 3. Comparison of ΔHRs with light conditions and dark conditions at 5 minutes after Ca channel blockers treatment. Panels A, B and C show the results of nicardipine, diltiazem and verapamil, respectively. The open column and the dotted column show the light conditions and the dark conditions, respectively. Each value represents the mean±S.E.M. for 5 embryos. *P<0.05, significantly different from another lighting condition on each dose group by t-test.

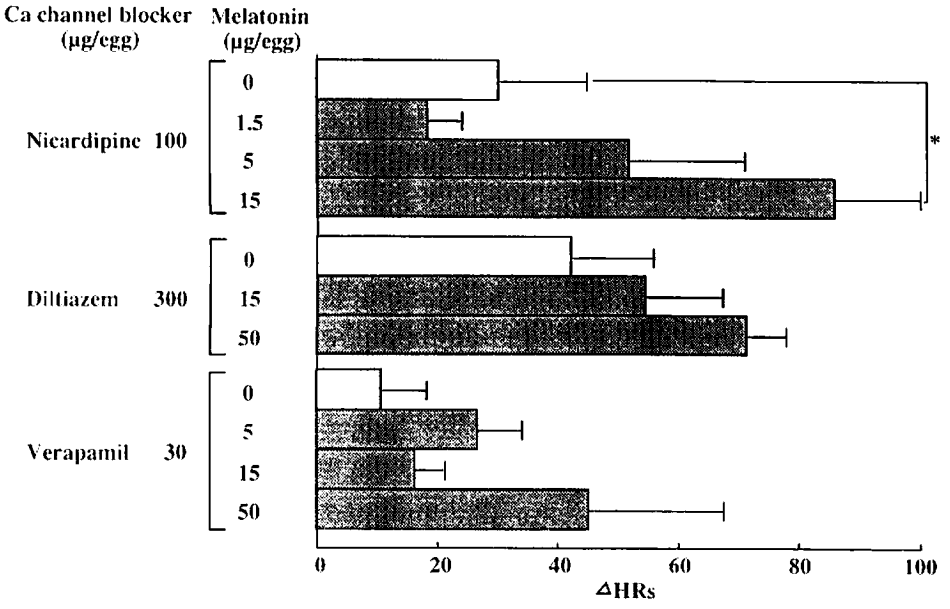

Fig. 4. Comparison of ΔHRs with Ca channel blocker alone and concomitant injection of melatonin at 5 minutes after injection of each compound. The open column and the dotted column show alone and concomitant injection, respectively. Each value represents the mean±S.E.M. of 5 embryos. *P<0.05, significantly different from 0 μg/egg dose group by Dunnett's test.

Discussoin

Nicardipine, diltiazem and verapamil are all Ca channel blockers that work on the electric potential - dependent L - type Ca channels (Watson and Girdlestone 1994). From a clinical viewpoint, diltiazem and verapamil are classified as Type I, which suppresses atrioventricular conduction at vasodilating doses, while nicardipine is classified as Type II, which shows dominant effects on the blood vessels (Shingh 1986, Taira 1987). Since L-type Ca channels in the chick embryonic heart are developed on day 11 of incubation (Brotto and Creazzo 1996), the changes observed in the ECG pattern were considered to be due to the effects of these Ca channel blockers. All these blockers caused bradycardia and arrhythmia which were considered due to the suppression of atrioventricular conduction since the type of arrhythmia was A-V block. Furthermore, nicardipine showed the weakest effect on the heart as previously reported with mammalian (Motomura and Hashimoto 1990, Taira 1987). This finding suggests the usefulness of chick embryos in this field.

Comparison of ECG patterns observed with chick embryos under light conditions (450 Lux) and dark conditions (12Lux) showed that all 3 compounds had higher effects on the cardiovascular systems under dark conditions than under light conditions. The hypotensive effects of these compounds in humans are reported to be more marked in the daytime, probably due to the fact that their plasma concentrations are higher in the daytime than in the nighttime (Gould et al. 1982A, Gould et al. 1982B, Lemmer et al. 1989). However, these differences observed after oral administration are considered to be due to differences in absorption in the daytime and in the nighttime. It is well known that administration into the air

sac of fertile egg is equivalent to intravenous administration and is not affected by absorption. This may be the reason for the different responses in chick embryos and humans. The validity of this hypothesis can be tested by measuring blood concentrations in chick embryos.

Concomitant injection of melatonin, a hypnotic agent biosynthesized in the pineal body and Ca channel blockers under light conditions, reproduced the results obtained under dark conditions. This finding strongly suggests that melatonin was responsible for the more marked effects of the Ca channel blockers on the ECG under dark conditions. While the contents of melatonin in the pineal body in chicks are known to be low in the daytime and high in the nighttime and to decrease when exposed to light (Sun et al. 1993), circadian rhythm of melatonin in chick embryos is still unknown. However, the pineal body of chick embryos develops in the roof of the third ventricle on day 3 of incubation (Calvo and Boya 1978) and shows a histopathological form characterizing the pineal body with melatonin secretory cells on day 10 of incubation (Calvo and Boya 1979). Furthermore, the activity of hydroxyindole - *o* - methyl transferase, an enzyme which exists only in the pineal body and is involved in the biosynthesis of melatonin is observed in chick embryos from day 12 of incubation (Wainwright 1974). These findings suggest that melatonin was biosynthesized and functioning in the chick embryos used in our experiments. It can therefore be concluded that chick embryos are useful for chronopharmacological studies and studies on drug interactions related to circadian rhythm in which melatonin is thought to play a role.

References

Brotto, M.A. and Creazzo, T.L. (1996) Ca2+ transients in embryonic chick heart: contribution from Ca^{2+} channels and the sarcoplasmic reticulum., *Am.J.Physiol.*, **270**(2 Pt 2), H518- 525.

Calvo, J and Boya, J. (1978) Embryonic development of the pineal gland of the chicken (Gallus gallus), *Acta Anat.*, **101**, 289-303.

Calvo, J and Boya, J. (1979) Ultrastructural study of the embryonic development of the pineal gland of the chicken(Gallus gallus), *Acta Anat.*, **103**, 39-73.

Gould, B.A., Hornung, R.S., Mannm, S., Balasubramanian, V. and Raftery, E.B. (1982A) Slow channel inhibitor verapamil and nifedipine in the management of hypertension, *J.Cardiovasc.Pharmacol.*, **4**, 5369-5373.

Gould, B.A., Mann, S., Keiso, H., Balasubramanian, V. and Raftery, E.B. (1982B) The 24- hour ambulatory blood pressure profile with verapamil., *Circulation*, **65**, 22-27.

Karnofsky, D.A. (1955) Investigation of diverse systems for cancer chemotherapy screening. *Cancer Res.* **15**, 83-88.

Lemmer, B., Behne, S. and Beckers, H.J. (1989) Chronopharmacology of oral nifedipine in healthy subjects, IVth Would Conference Clinical Pharmacology Therapeutics, Manheim Heidelberg, Abstr.

Miyazaki, H., Sugiyama, T., Saito, K., Kubota, N., Yoshiyama, Y. and Shimada, H. (1994) Toxicological study of cardiovascular drugs in chick embryos and rodents, *In vitro Toxicology*, **7**(3), 243-246.

Motomura, S. and Hashimoto, K. (1990) Reconsideration of vascular selectivity of dihydropyridine calcium antagonists: comparison of cardiovascular profile of mepirodipine, a novel dihydropyridine consisting of a single stereoisomer with (+)-(S)-(S) conformation, with those nifedipine and nicardipine, *Jpn.J.Pharmacol.*, **52**, 319-330.

Shepard, T.H. (1976): Teratogenic Agents, 2nd ed. John Hopkins University Press, Baltimore.

Shingh, B.N. (1986) The mechanism of action of calcium antagonists relative to their clinical- applications, *Br.J.Clin.Pharmacol.*, **21**, 109S-121S

Sugiyama, T., Miyazaki, H., Saito, K., Kubota, N., Shimada, H. and Miyamoto, K. (1996) Chick embryos as an alternative experimental animal for cardiovascular investigations: stable recording of electrocardiogram of chick embryos in ovo on the 16th day of incubation, *Toxicol.Applied Pharmacol.*, **138** 262-267.

Sugiyama, T., Miyazaki, H., Saito, K. and Shimada, H. (1997) The pharmacological effect of β-blocker can be evaluated by using a chick embryo tachycardia model, in "*Animal Alternatives, Welfare and Ethics*", ed. by L.F.N. van Zutphen and M. balls, pp. 909-911, Elsevier Science B.V., Amsterdam.

Sun, J.H, Reiter, R.J., Hattori, A., Yaga, K., Herbert, D.C. and Tsin, A.T.C. (1993) Phototransduction-related circadian changes in indoleamine metabolism in the chick pineal gland in vivo, *J.Pineal*

Res., **15**, 132-137.

Taira, N. (1987) Differences in cardiovascular profile among calcium antagonists. *Am.J.Cardiol.*, **59**, 24B-29B.

Yoshiyama, Y., Sugiyama, T., Kubota, N., Miyazaki, H., Saito, K., Tomonaga, F., Shimada, H. and Ohdo, S. (1995) Manipulation of the lighting schedule can modify the pharmacological effects of theophylline in chick embryos, *Biol. Pharm. Bull.*, **18**(5), 776- 778

Wainwright, S.D. (1974) Course of the increase in hydroxy-indole-O-methyl transferase activity in the pineal gland of the chick embryo and young chick, *J.Neurochem.*, **22**, 193- 196.

Watson, S. and Girdlestone, D.(1994) Tips Receptor and Ion Channel Nomenclature Supplement 1994 In Trends Pharmacol.Sci. 5th ed. p43