Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 405-412

ACTIVATION OF THE VOLTAGE-DEPENDENT Ca^{2+} CHANNEL IN RAT HEART CELLS BY DIHYDROPYRIDINE DERIVATIVES

Jean-François Renaud, Jean-Pierre Méaux, Georges Romey, Annie Schmid and Michel Lazdunski

Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06034 NICE CEDEX, France

Received October 24, 1984

SUMMARY. The two dihydropyridines Bay K8644 and CGP 28392 increase $^{45}Ca^{2+}$ influx in cultured rat cardiac cells with half-maximal effects at 2 nM and 30 nM respectively at a membrane potential of -75 mV. This stimulation of Ca^{2+} uptake is inhibited by nitrendipine, verapamil and bepridil. Ca^{2+} channel activation produced by Bay K8644 and CGP 28392 has been compared with Ca^{2+} channel activation produced by depolarization. There is no addition between the effects of drugs activating the Ca^{2+} channel and the effects of depolarization suggesting that Bay K8644 and CGP 28392 work preferentially on polarized membranes. $^{45}Ca^{2+}$ flux experiments yielded results which are in excellent agreement with electrophysiological and contraction data obtained with the same cells in culture. Dose-response curves for the physiological effects of the drugs are observed over the same range of concentrations as their inhibition of [^{3}H]nitrendipine binding to its receptor.

INTRODUCTION. Voltage-dependent calcium channels are responsible for the plateau phase of the action potential of the cardiac cell. They are closely linked to contractile activation of myocardium. They are blocked by compounds known as calcium antagonists which suppress the contractile performance of the heart (1-3). These compounds include structurally different groups of drugs such as dihydropyridine including nitrendipine, nimodipine or nisoldipine and other compounds like verapamil, D_{600}, diltiazem and bepridil.

It has been shown recently that relatively small structural modifications of dihydropyridine molecules which block Ca^{2+} channels can lead to a novel class of drugs such as Bay K8644 and CGP 28392 which bind to cardiac membranes with high affinity (4), induce an increase in cardiac and smooth muscle contractility (5, 6), enhance Ca^{2+} influx in human platelets and in neuroblastoma-glioma cells (7, 8), enhance secretion in a pituitary cell line (9) and activate chromaffin cell Ca^{2+} channels (10).

0006-291X/84 $1.50

The present work reports the properties of interaction of Bay K8644 and CGP 28392 with Ca^{2+} channels in rat heart cells in culture using $^{45}Ca^{2+}$ flux, electrophysiological and binding studies.

MATERIALS AND METHODS

Cell culture. Dissociation of ventricles from 2 to 3 day-old rats into single cells was carried out as previously described (11). After dissociation, the dispersed cells were plated at a density of 4×10^5 cells/cm^2 and cultured as monolayers using both 35 mm diameter Petri dishes (Corning) and 24-well tissue culture plates (Nunc). Cultures were maintained at 37°C in a water-saturated atmosphere of air/CO_2 (95/5). The medium was changed once, two days after plating, and monolayers were used 3 to 7 days after plating for both electrophysiological and $^{45}Ca^{2+}$ influx studies.

Tissue and membrane preparation. Hearts from Wistar albino rats were used at adult stage (3 to 4 month-old). Rats were killed by decapitation and venticles were quickly removed and washed in an ice-cold oxygenated solution containing 20 mM Tris/Cl 0.25 M sucrose and 1 mM EDTA at pH 7.4 (TSE buffer) and homogenized at setting 5 with a Polytron apparatus using three 5-sec bursts separated by 30-sec pauses. All subsequent operations leading to the plasma membrane fraction (P_3) were carried out at 4°C according to Paris et al. (12). The cardiac plasma membrane fraction (P_3) was kept in TSE buffer at a protein concentration of approximately 5 mg/ml in liquid nitrogen until used for binding studies.

Binding assays. Binding assays were carried out as previously described (13). 400-500 µg of plasma membrane fraction (P_3) were equilibrated in 1 ml of the standard incubation medium containing 20 mM Tris/Cl, 50 mM choline chloride and 10^{-4} M of the protease inhibitor phenyl methylsulfonyl fluoride at pH 7.4 and 4°C. The effect of Ca^{2+} channel blockers and activators on specific [^{3}H]nitrendipine binding to Ca^{2+} channels in the heart plasma membrane fraction was measured under equilibrium conditions at 4°C during 1 hr using a fixed concentration of [^{3}H]nitrendipine and increasing concentrations of unlabeled drugs. All experiments were carried out under dim light.

$^{45}Ca^{2+}$ flux experiments. The determination of nitrendipine-sensitive and -insensitive rates of $^{45}Ca^{2+}$ uptake in the presence or absence of Ca^{2+} agonists by cardiac cells in culture was carried out in 24-well tissue culture plates (Nunc). The cells were cultured between 4 and 7 days before flux measurements were performed. The rates of $^{45}Ca^{2+}$ flux were determined in media containing K^+ at concentrations ranging between 5 and 50 mM with a corresponding decrease either in Na^+ (or Li^+ in some experiments) concentrations from 140 to 90 mM. The determination of rates of $^{45}Ca^{2+}$ uptake first involved a preincubation of the cells with the desired concentration of the drug to be studied for 15 min at 37°C in a medium containing adequate concentrations of Na^+ and K^+, 5 mM glucose and 25 mM Hepes-Tris at pH 7.4 in the presence of 0.2 mM EGTA. The medium was then removed by aspiration and the cells were incubated in a new medium free of EGTA and supplemented with 1.8 mM $CaCl_2$ and 2 µCi/ml of $^{45}Ca^{2+}$ in the presence of the same concentrations of cations and drugs that were used in the preincubation medium. Kinetics of $^{45}Ca^{2+}$ uptake were followed by removing the medium at different times at 37°C. Initial rates of $^{45}Ca^{2+}$ uptake were routinely measured after 20 sec to obtain dose-response curves for Ca^{2+} agonists and Ca^{2+} antagonists. At the end of the incubation, wells were washed three times in less than 10 sec with 3 ml of washing medium containing 140 mM choline, 1 mM $LaCl_3$ and 25 mM Hepes-Tris at pH 7.4. After the third wash, 2 ml of 0.1 N NaOH were added to each well and the radioactivity incorporated by the cells was counted. Protein concentrations were measured according to Hartree (14).

Electrophysiology and contraction. Electrical recordings from new-born rat heart cells cultured in the form of monolayers have been carried out as previously described (15). Spontaneous beating rates and amplitude of contractions were recorded simultaneously with the membrane voltage recording and analysed with the combination of a Sony camera and a simplified real time TV image analyser based on an Apple II microcomputer (15). With this system the recorded images are successively compared to a reference image and the number of "pixels" that have changed state are determined. This latter value is fed into a D/A converter in order to follow the deformation of cells in real time on an oscilloscope.

RESULTS

The inset of Fig. 1 shows the time-course of $^{45}Ca^{2+}$ uptake by cardiac cells that have been incubated in a low K+ medium containing 5 mM KCl and in a high K+ medium containing 40 mM KCl in the presence and/or absence of 10 nM Bay K8644 and 1 μM nitrendipine. When cells are in a low K+ medium their membrane potential is at -75 ± 5 mV and $^{45}Ca^{2+}$ uptake is not affected by nitrendipine. Addition of 10 nM Bay K8644 increases the initial rate of $^{45}Ca^{2+}$ uptake (2.5-fold). This increased $^{45}Ca^{2+}$ uptake is blocked by nitrendipine (1 μM) (Fig. 1A and inset). When cells are incubated in a high K+ medium they are depolarized to -30 ± 5 mV (not shown). Depolarization with K+ greatly increases the initial rate of $^{45}Ca^{2+}$ uptake (4-fold) and this increased uptake is inhibited by 1 μM nitrendipine (Fig. 1B and inset). Addition of 10 nM Bay K8644 does not increase the component of $^{45}Ca^{2+}$ uptake induced by depolarization and does not prevent its inhibition by nitrendipine (Fig. 1B and inset). $^{45}Ca^{2+}$ uptake induced both by Bay K8644 under polarized conditions, and by depolarization (40 mM KCl), is not due to

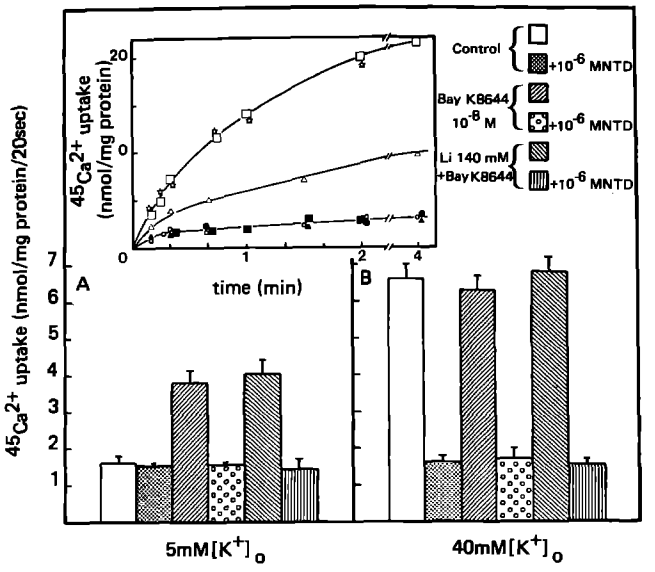

Fig. 1 (inset) : Time-course of $^{45}Ca^{2+}$ uptake in 5 or 40 mM $[K^+]_o$. Cardiac cells from new-born rats were cultured and pre-incubated as described in Materials and Methods. Uptake of $^{45}Ca^{2+}$ was measured for increasing periods of time in medium containing 2 μCi/ml of $^{45}Ca^{2+}$ and either 5 mM (○,△,▲), or 40 mM (□,■,☆) K+, in the presence (△,☆,▲) or absence (□,■) of 10 nM Bay K8644 and in the presence of nitrendipine (NTD) (1 μM) (■,▲,○,●). Each point represents the mean value of 4 experiments. **Main panel A** : Effect of 10 nM Bay K8644 on $^{45}Ca^{2+}$ uptake under polarized conditions ($[K^+]_o$ 5 mM). Uptake of $^{45}Ca^{2+}$ was measured for 20 sec at 37°C under the indicated conditions. Each bar gives the mean value ± S.E. of 4 experiments. **Main panel B** : Effect of high $[K^+]_o$ on $^{45}Ca^{2+}$ uptake. Each bar gives the mean value ±S.E. of 4 experiments.

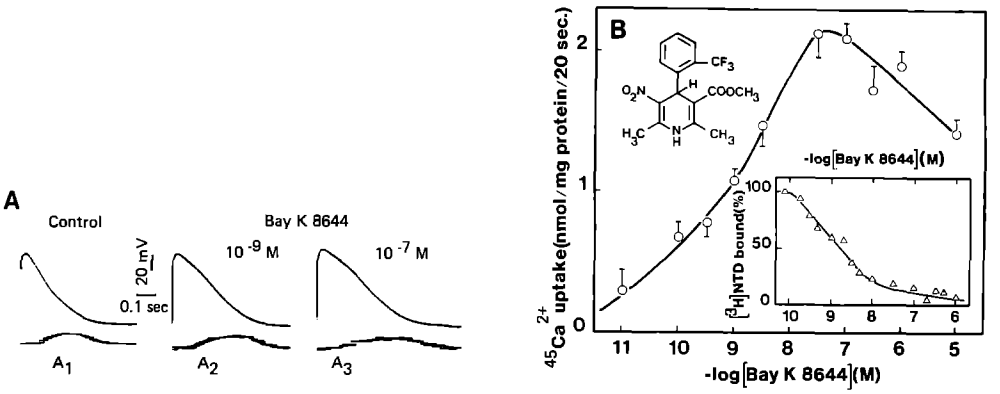

Fig. 2 : Effects of Bay K8644. (A) on electrical and mechanical activities of rat cardiac cells in culture. **(A_1)** Control action potential, **(A_2)** 10 min after addition of 1 nM Bay K8644, **(A_3)** 10 min after addition of 100 nM Bay K8644 to the same cell. The lower trace shows the contraction. **(B)** Stimulation of the initial rate of $^{45}Ca^{2+}$ uptake in low $[K^+]_o$ medium (o) by increasing concentrations of Bay K8644. **Inset** : Effect of Bay K8644 on specific [^{3}H]nitrendipine (0.3 nM) binding. Curves represent the mean values of 4 series of experiments.

the Na^+/Ca^{2+} exchange system since a similar nitrendipine-sensitive $^{45}Ca^{2+}$ uptake component is found when Na^+ is replaced by Li^+ (Fig. 1A and B). Li^+ cannot substitute for Na^+ in the Na^+/Ca^{2+} exchange system.

The effects of Bay K8644 on both the action potential and contraction of new-born heart cells cultured in monolayers are shown Fig. 2A. Bay K8644 in concentrations between 10^{-9} M and 10^{-7} M produces marked effects on both the duration of the action potential and the contraction. The action potential duration measured at 50% repolarization (APD_{50}) is increased by as much as 66% (10^{-9} M Bay K8644) to 86% (10^{-7} M Bay K8644) as compared to the control value (150 msec). Bay K8644 enhances the duration of contraction in the same range of concentrations over which changes in the action potential duration are observed (Fig. 2A). The dose-response curve for the action of Bay K8644 on the initial rate of $^{45}Ca^{2+}$ uptake is shown Fig. 2B. The half-maximal effect of Bay K8644 on $^{45}Ca^{2+}$ uptake stimulation is observed at 2 nM. The inset of Fig. 2B shows that increasing concentrations of Bay K8644 gradually inhibit specific [^{3}H]nitrendipine binding to cardiac membranes with a half-maximum effect near 2 nM. CGP 28392 behaves similarly to Bay K8644. The electrophysiological effects of CGP 28392 are shown in Fig. 3A. CGP 28392 increases APD_{50} by 23% and 55% at concentrations of 10^{-8} M and 10^{-7} M respectively. As previously observed for Bay K8644, CGP 28392 also enhances contractility in parallel with its effect on Ca^{2+}

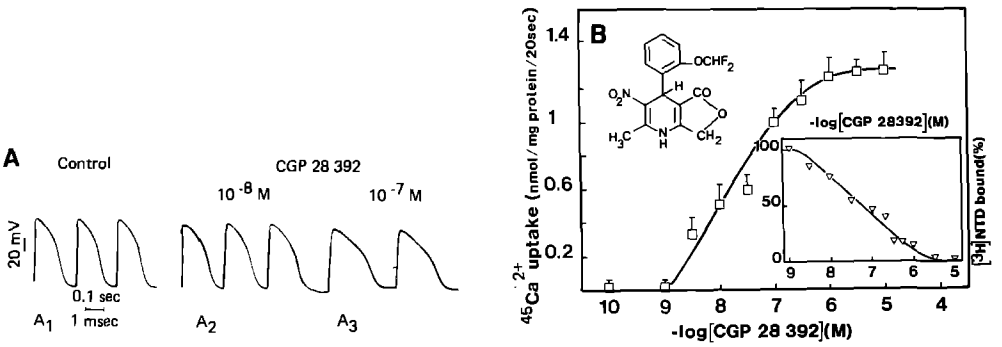

Fig. 3 : Effects of CGP 28392. (A) on electrical activity of new-born rat cardiac cells in culture. **(A_1)** Control action potential, **(A_2)** 10 min after addition of 10 nM CGP 28392, **(A_3)** 10 min after addition of 100 nM CGP 28392 to the same cell. **(B)** Stimulation of the initial rate of $^{45}Ca^{2+}$ uptake in low $[K^+]_o$ medium (□) by increasing concentrations of CGP 28392. **Inset :** Inhibition of [^{3}H]nitrendipine (0.3 nM).

channel activity (not shown). The dose-response curve of the increased rate of $^{45}Ca^{2+}$ uptake due to CGP 28392 is presented in Fig. 3B; the half-maximum effect is observed at 30 nM. CGP 28392 also inhibits [^{3}H]nitrendipine binding to cardiac membranes with a half-maximal effect at 50 nM (inset Fig. 3B).

Fig. 4 shows dose-response curves for the action of two Ca^{2+} channel blockers which do not belong to the dihydropyridine series. Verapamil and bepridil decrease the component of initial rate of $^{45}Ca^{2+}$ uptake due either to the action of 10 nM Bay K8644 under polarized conditions (Fig. 4A), or to depolarization (40 mM K^+) in the absence of Bay K8644 (Fig. 4B). Half-maximum inhibition for verapamil and bepridil respectively

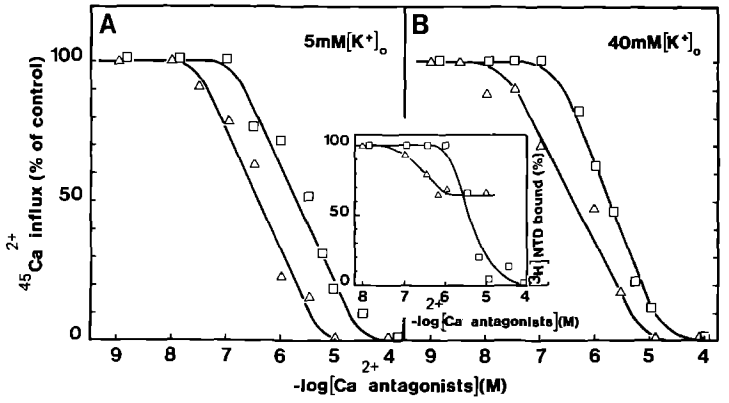

Fig. 4 : Verapamil and bepridil-sensitive rate of $^{45}Ca^{2+}$ uptake. Main panels : Dose-response curves for verapamil (△) and bepridil (□) effects on the initial rate of $^{45}Ca^{2+}$ uptake in the presence of 10 nM Bay K8644 **(A)** in low $[K^+]_o$ medium. Effects in high $[K^+]_o$ medium in the absence of Bay K8644 **(B). Inset :** Effects of verapamil (△) and bepridil (□) on [^{3}H]nitrendipine (0.3 nM) binding to cardiac membranes. Curves represent the mean values of 6 series of experiments.

are found at 350 nM and 1 μM in the presence of 10 nM Bay K8644 under polarized conditions and at 400 nM and 2 μM under depolarized conditions. Inset in Fig. 4 shows the effects of increasing concentrations of verapamil and bepridil on [^{3}H]nitrendipine binding to cardiac membranes. Half-maximum inhibition values for verapamil and bepridil are 250 nM and 8 μM respectively.

DISCUSSION. The two dihydropyridine derivatives, Bay K8644 and CGP 28392, which have been studied in this paper have the following properties of interaction with the Ca^{2+} channel in cultured rat cardiac cells : (i) They both activate $^{45}Ca^{2+}$ entry when the cardiac cell is polarized at -75 mV. (ii) Activation of Ca^{2+} channels is observed at relatively low concentrations of the drugs, the order of potency being Bay K8644 (ED_{50} = 2 nM) > CGP 28392 (ED_{50} = 30 nM). (iii) Bay K8644 and CGP 28392 are unable to increase the probability of opening of Ca^{2+} channels under depolarizing conditions. Their effects are not additive with those of depolarization. This observation strongly suggests that the drug action is voltage-dependent, being more efficient at -75 mV than at -30 mV. (iv) Drug concentrations at which the stimulation of $^{45}Ca^{2+}$ influx is observed are in satisfactory correspondence with those which produce an inhibition of [^{3}H]nitrendipine binding. (v) Ca^{2+} channel blockers like verapamil and bepridil, which do not belong to the dihydropyridine series and which bind to a site distinct from that of dihydropyridines (3, 16), block with a similar potency both Ca^{2+} channels activated by depolarization of the membrane to -30 mV or activated by Bay K8644. (vi) Ca^{2+} flux data measured in the presence of Bay K8644 and CGP 28392 are consistent with electrophysiological findings. Prolongation of the plateau phase of the action potential which is due to Ca^{2+} channel activity is seen in the same range of drug concentrations over which an increased rate of $^{45}Ca^{2+}$ uptake is observed. This prolongation of the action potential is parallelled by an increased contractility as expected for an increased Ca^{2+} entry.

All these results are consistent with very recent patch-clamp and voltage-clamp experiments which have shown that both Bay K8644 (17-19) and CGP 28392 (17) increase the probability of finding the cardiac Ca^{2+} channel in the open form (17-19). The drugs probably act by promoting a long-lasting open state of the Ca^{2+} channel (17,

19). The dose-response curve observed for Bay K8644 by patch-clamp techniques (17) is very similar (ED_{50} = 5-10 nM) to the one found in this study using $^{45}Ca^{2+}$ flux.

Rat cardiac cells in culture have a maximal capacity for [^{3}H]nitrendipine binding of 60 fmol/mg protein which corresponds to about 10^4 sites/cell (not shown). Ca^{2+} influx values measured at saturating concentrations of Bay K8644 and CGP 28392 are 2.2 and 1.4 nmol/mg protein/20 sec, respectively (Fig. 2 and 3). If we assume a one-to-one ratio between nitrendipine binding sites and Ca^{2+} channels, our data indicate a Ca^{2+} influx of 1.9×10^3 and 1.2×10^3 Ca^{2+} ions transported/sec/channel site at saturating concentrations of Bay K8644 and CGP 28392 respectively. Under our conditions, the action of Bay K8644 and CGP 28392 would cause the opening of 5 to 10 channels per cardiac cell at any given time assuming an elementary Ca^{2+} current of about 1 pA (20).

Because both Bay K8644 and CGP 28392 elicit a marked increase in the rate of $^{45}Ca^{2+}$ influx under polarized conditions one can easily foresee that these compounds will become valuable tools in Ca^{2+} channel studies. This situation would be similar to that of sea anemone toxin which activates Na^{+} channels in heart and other excitable tissues (21).

ACKNOWLEDGEMENTS. We wish to thank Dr Perin from Bayer Pharma (France) for the gift of nitrendipine, Mrs A.M. Lamy from Biosedra (France) for verapamil, Dr Busch for bepridil, Dr H. Glossmann for CGP 28392, and Dr H. Glossmann and Dr M. Schramm (Bayer) for Bay K8644. Thanks are due to M.T. Ravier and C. Roulinat-Bettelheim for expert technical assistance. This work was supported by the Centre National de la Recherche Scientifique and the Fondation pour la Recherche Médicale.

REFERENCES

1. Tsien, R.W. (1983) Annu. Rev. Physiol., 45, 341-358.
2. Fleckenstein, A., and Fleckenstein-Grün, G. (1984) Physiology and Pathophysiology of the Heart, pp. 421-442, Sperelakis, N. (ed.), Martinus Nijhoff Publishing Boston/The Hague/Dordrecht/Lancaster.
3. Triggle, D.J., and Janis, R.A. (1984) Modern Methods in Pharmacology, pp. 1-28, Alan R. Liss, Inc., N.Y.
4. Belleman, P. (1984) F.E.B.S., 167, 88-92.
5. Schramm, M., Thomas, G., Towart, R., and Franckowiak, G. (1983) Nature, 303, 535-537.
6. Vaghy, P.L., Grupp, I.L., Grupp, G., and Schwartz, A. (1984) Circulation Research (in press).
7. Erne, P., Burgisser, E., Buhler, F.R., Dubach, B., Kühnis, H., Meier, M., and Rogg, H. (1984) Biochem. Biophys. Res. Commun., 118, 842-847.
8. Freedman, S.B., and Miller, R.J. (1984) Proc. Natl. Acad. Sci. USA, 81, 5580-5583.

9. Tan, K.N., and Tashjian, A.H. (1984) J. Biol. Chem., 259, 418-426.
10. Garcia, A.G., Sala, F., Reig, J.A., Viniegra, S., Frias, J., Fonteriz, R., and Gandia, L. (1984) Nature, 309, 69-71.
11. Renaud, J.F., Kazazoglou, T., Lombet, A., Chicheportiche, R., Jaimovich, E., Romey, G., and Lazdunski, M. (1983) J. Biol. Chem., 258, 8799-8805.
12. Paris, S., Fosset, M., Samuel, D., and Ailhaud, G. (1977) J. Moll. Cell. Cardiol., 9, 161-174.
13. Renaud, J.F., Kazazoglou, T., Schmid, A., Romey, G., and Lazdunski, M. (1984) Eur. J. Biochem., 139, 673-681.
14. Hartree, E.F. (1972) Anal. Biochem., 48, 422-427.
15. Bordes, M., Bernengo, J.C., and Renaud, J.F. (1982) Rev. Sci. Instrum., 54, 1053-1058.
16. Ferry, D.R., and Glossmann, H. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol., 321, 80-83.
17. Kokubun, S., and Reuter, H. (1984) Proc. Natl. Acad. Sci. USA, 81, 4824-4827.
18. Sanguinetti, M.C., and Kass, R.S. (1984) J. Mol. Cell. Cardiol., 16, 667-670.
19. Hess, P., Lansman, J.B., and Tsien, R.W. (1984) Nature, in press.
20. Reuter, H. (1983) Nature, 301, 569-574.
21. Lazdunski, M., and Renaud, J.F. (1982) Annu. Rev. Physiol., 44, 463-473.