Biochemical Pharmacology, Vol. 40, No. 5, pp. 955–959, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

EFFECTS OF THE NOVEL DIHYDROPYRIDINE DERIVATIVE NIGULDIPINE ON THE CYTOPLASMIC FREE CALCIUM CONCENTRATION OF MOUSE THYMOCYTES

MICHAEL P. R. DROZD* and KLAUS GIETZEN

Department of Pharmacology and Toxicology, University of Ulm, Oberer Eselsberg, D-7900 Ulm/Donau, Federal Republic of Germany

(*Received* 7 *September* 1989; *accepted* 30 *March* 1990)

Abstract—Niguldipine, a novel dihydropyridine derivative, was tested for its effects on the cytoplasmic free Ca^{2+} concentration of mouse thymocytes. In quin-2-loaded cells, a concentration-dependent rise of cytoplasmic Ca^{2+} can be detected, which requires extracellular Ca^{2+}. The effect of niguldipine reaches a maximum after about 5 min; a similar time course has been observed, when using concanavalin A as a stimulus. Niguldipine provokes influx of Ca^{2+} into thymocytes, but not of Mn^{2+}. Moreover, the effect of niguldipine exhibits some degree of stereospecificity, since (−)-niguldipine was more effective than its (+)-enantiomer. The action of niguldipine could be reversed by addition of bovine serum albumin, but not by addition of nitrendipine. None of several agents tested (e.g. felodipine, nitrendipine, trifluoperazine, cloxacepride, phenylephrine and ouabain) could mimic the effect of niguldipine at a concentration of 1 μM.

Dihydropyridine (DHP†) derivatives are commonly used in the therapy of cardiovascular diseases and in addition as valuable research tools [1]. Their most striking feature is their affinity for the voltage-operated Ca^{2+} channel. Depending on the substance, binding to the DHP-binding site can result in blockade or activation of the channel [2–4]. Therefore DHP effects are mostly associated with the Ca^{2+} second messenger system. Niguldipine is a new DHP derivative with antihypertensive activity in animal models [5]. It binds to the DHP receptor on L-type channels and moreover to the α_1-adrenoceptor subtype [6]. Beside this, niguldipine is a potent calmodulin antagonist (Gietzen *et al.*, unpublished data). In contrast to other potent and membrane permeable calmodulin antagonists like trifluoperazine or felodipine, niguldipine has no autofluorescence and thus causes less problems in fluorescence assays as the measurement of cytoplasmic free Ca^{2+} with quin-2. To examine the effects of this new interesting substance on the Ca^{2+} second messenger system, we choose mouse thymocytes as a cellular model. In the present paper, we show that niguldipine can reversibly increase cytoplasmic free Ca^{2+} in these cells.

MATERIALS AND METHODS

Materials. Racemic niguldipine (B844-39), the (+)- and (−)-enantiomers (B859-34 and B859-35, respectively) and nitrendipine were obtained from Byk Gulden-Lomberg GmbH (Konstanz, F.R.G.). Cloxacepride was provided by Merckle GmbH (Ulm, F.R.G.). Con A (Type V), BSA (Fraction V), DTPN and quin-2/AM were from Sigma (Heidelberg, F.R.G.) and Bay K 8644 from Calbiochem (Frankfurt, F.R.G.). Niguldipine, nitrendipine, felodipine and cloxacepride were dissolved in and diluted with DMSO. From these stock solutions, aliquots were added to cell suspensions to give a final DMSO concentration of 0.1% (v/v). Con A and DTPN were dissolved in Hepes-buffered saline as used for cell suspensions (see below).

Animals. Male NMRI outbred mice, 3 to 6 weeks old, were obtained from the Zentralinstitut fuer Versuchstierzucht (Hannover, F.R.G.).

Cells. Animals were killed and thymus glands were teased in RPMI 1640 Medium (Gibco) containing 10% heat inactivated fetal calf serum (RPMI-FCS). Cells were suspended by gently pipetting and washed twice in RPMI-FCS. Cells were diluted to 20×10^6 cells/mL and kept in petri dishes at room temperature. Cell viability, as checked by trypan blue dye exclusion, was more than 98%. As a more reliable criterion we used the ability of viable thymocytes to respond to 10 μg Con A/mL with an $[Ca^{2+}]_i$ increase to more than 200 nM.

$[Ca^{2+}]_i$ *measurement*. For dye loading aliquots of the cell suspension were incubated with 20 μM quin-2/AM for 30 min at 37°. Then the cells were washed twice with RPMI-FCS and resuspended at 2×10^6 cells/mL in a buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 10 mM Hepes (pH 7.35) and 1 g glucose/mL and stored at room temperature until use. RPMI-FCS adhering to the cells did not influence the measurement. Continuous fluorescence measurement was carried out with a Shimadzu RF-540 spectro-

* To whom correspondence should be addressed.

† Abbreviations: *AF*, autofluorescence; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; Con A, concanavalin A; DHP, dihydropyridine; DMSO, dimethylsulfoxide; DTPN, diethylenetriaminepentaacetic acid; EGTA, ethyleneglycolbis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; quin-2/AM, quin-2-acetoxymethylester; RPMI-FCS, RPMI 1640 Medium containing 10% (v/v) fetal calf serum.

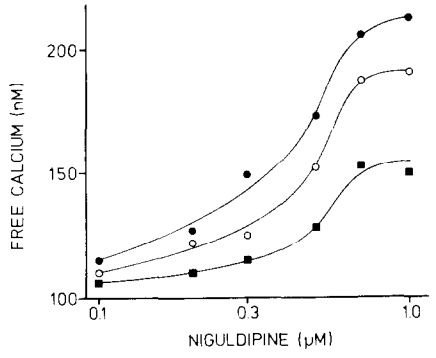

Fig. 1. Concentration-dependent increase of $[Ca^{2+}]_i$ 5 min after addition of a single dose of racemic niguldipine (○, B844-39, (−)-niguldipine (●, B859-35) and (+)-niguldipine (■, B859-34). The symbols represent mean values of 4 to 8 different preparations. SD was in the range of 7 to 17%.

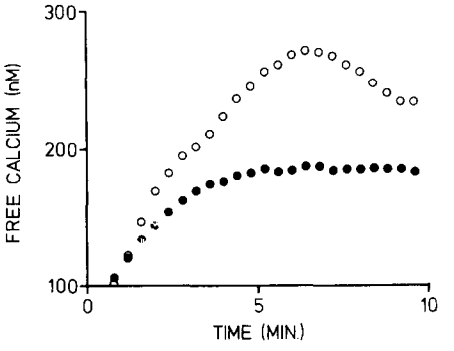

Fig. 2. Time course of $[Ca^{2+}]_i$ after addition of 1 μM racemic niguldipine (B844-39, ●) and 10 μg/mL Con A (○). The symbols represent mean values of 5 and 4 different preparations for niguldipine and Con A, respectively.

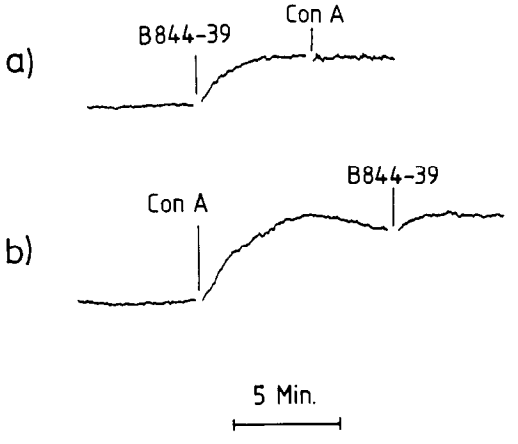

Fig. 3. Time course of the quin-2 fluorescence. (a) Subsequent addition of 1 μM racemic niguldipine (B844-39) and 10 μg/mL Con A. Subsequent addition of two doses of 1 μM B844-39 results in exactly the same time course and was therefore not shown. (b) Subsequent addition of 10 μg/mL Con A and 1 μM B844-39.

fluorimeter in a thermostatted pyrex glass cuvette at 37° and constant moderate stirring. Excitation and emission wavelengths were 339 nm (slit 2 nm) and 495 nm (slit 10 nm), respectively. For some other measurements several samples were incubated outside the fluorimeter and were only stirred during the fluorescent measurement (results in Fig. 7). Substances were added after preincubation of the cells for 5 min at 37°. Controls were treated in the same way, except that DMSO was added instead of test substances dissolved in DMSO. However, DMSO alone was without effect. F_{max} was determined after cell lysis with 0.025% (w/v) Triton X-100 in the presence of 15 μM DTPN. Autofluorescence (AF) of the cell suspension was measured after dye quenching with 1 mM $MnCl_2$. $[Ca^{2+}]_i$ was calculated as described in Ref. 7:

$$[Ca^{2+}]_i = K_D \frac{F - F_{min}}{F_{max} - F}$$

where $K_D = 115$ nM, and $F_{min} = 1/6(F_{min} - AF) + AF$.

Statistics. Significance was tested with the Student's t-test for unpaired samples.

RESULTS

In Ca^{2+}-containing buffer, racemic niguldipine (B844-39) caused a concentration dependent increase of the fluorescence signal corresponding to cytoplasmic free Ca^{2+} concentrations with a maximum of approximately 200 nM (Fig. 1) in mouse thymocytes. At all tested concentrations, the (−)-enantiomer B859-35 raised $[Ca^{2+}]_i$ to higher concentrations than the (+)-enantiomer B859-34. This difference is significant ($P < 0.01$) for niguldipine concentrations ≥300 nM. At about 1 μM, the curves for racemic niguldipine and its (+)-enantiomer reach a plateau. For (−)-niguldipine, a plateau was not completely reached within the concentration range shown in Fig. 1. However, above 1 μM niguldipine, we obtained no reproducible results, which we attribute to the reduced solubility of the hydrophobic test substances at high concentrations. Anyhow, it is apparent that the lower response to the (+)-enantiomer does not result from a shift of the dose–response curve to higher concentrations.

After a single dose of 1 μM B844-39, the Ca^{2+} concentration reached its maximum within 5 min and then remained constant over the observed time period (Fig. 2). During this plateau phase, a second dose of 1 μM B844-39 (data not shown) or addition of Con A (10 μg/mL) did not alter the Ca^{2+} signal (Fig. 3a). When using Con A (10 μg/mL) as stimulus $[Ca^{2+}]_i$ was raised to higher concentrations than with B844-39. The maximum appeared 7 min after the stimulus and was followed by a decrease in fluorescence signal (Fig. 2). Addition of 1 μM B844-39 to Con A-stimulated cells resulted in a further small increase in quin-2 fluorescence and hence in intra-

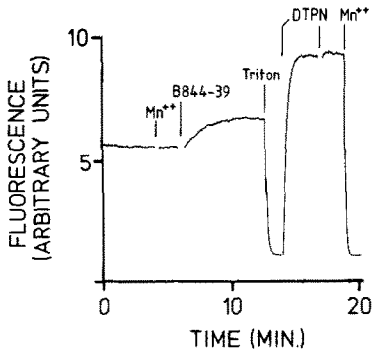

Fig. 4. Time course of quin-2 fluorescence signal in the presence of $MnCl_2$. Mn^{2+} was 50 μM (first dose) and 1 mM (second dose), B844-39 1 μM, Triton X-100 0.025%, DTPN each dose 150 μM. In the presence of Mn^{2+}, addition of B844-39 results in an increase of the fluorescence signal, although the Mn^{2+} concentration was sufficient to quench the quin-2-derived fluorescence signal completely after cell lysis with Triton X-100. In this way, an B844-39 induced influx of Mn^{2+} could have been detected by quenching of the fluorescence below the signal of resting cells. In this experiment F_{max} was measured by complexing Mn^{2+} of the first dose with DTPN, a chelator for heavy metal ions. The second dose of Mn^{2+} shows that already 50 μM Mn^{2+} that resulted from the first dose were sufficient to completely quench the quin-2 derived fluorescence, in order to obtain F_{min}.

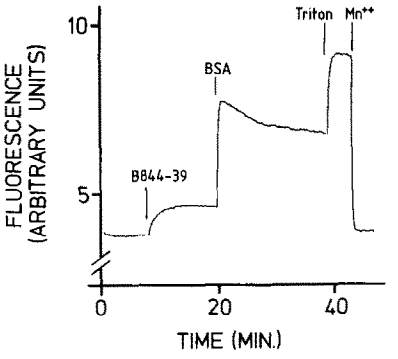

Fig. 5. Reversal of the niguldipine (1 μM B844-39) induced increase of quin-2 fluorescence by 0.2% (w/v) BSA. The initial rise of the signal after addition of BSA is due to an increase in autofluorescence. In contrast to Fig. 4, no Mn^{2+} was added before the other substances. Triton X-100 was added together with DTPN as described in Materials and Methods and thus results in an increase of fluorescence (F_{max}).

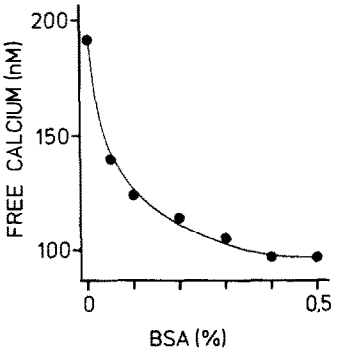

Fig. 6. Effect of BSA on the niguldipine induced increase of $[Ca^{2+}]_i$. BSA was added 6 min after B844-39. After another 6 min, $[Ca^{2+}]_i$ was determined. The symbols are mean values of at least 4 preparations. SD was in the range of 2 to 10%.

cellular Ca^{2+} concentration (Fig. 3b). Because of the varying response of cells from different animals and the lower precision of the quin-2 Ca^{2+} measurement at higher Ca^{2+} concentrations, the effects shown in Fig. 3 are demonstrated as two typical fluorescence protocols, each out of a series of at least five experiments.

In buffer containing 1 mM EGTA instead of 1 mM $CaCl_2$, the B844-39-induced increase of $[Ca^{2+}]_i$ was absent. Addition of 2 mM $CaCl_2$ after B844-39 increased cytoplasmic free Ca^{2+} from approximately 60–70 nM to the normal concentration around 100 nM. A further increase as shown in Figs 2 and 3a did not appear, although B844-39 was still present in the buffer (data not shown).

As an indicator for the influx of ions other than Ca^{2+}, we added $MnCl_2$ (50 μM) to the Ca^{2+}-containing cell suspension prior to stimulation with B844-39. Mn^{2+} entry into quin-2-loaded cells can be easily detected by a marked decrease in fluorescence intensity. In this way even a small Mn^{2+} flux could be seen, since the affinity of quin-2 for Mn^{2+} is much higher than for Ca^{2+} [8]. However, addition of Mn^{2+} did not alter the signal of resting cells or prevent a rise in fluorescence following the addition of B844-39, though the Mn^{2+} concentration was sufficient to quench quin-2-derived fluorescence completely after cell lysis with Triton X-100 (Fig. 4). Only when the measurement exceeded about 15 min, a slow decrease in fluorescence was seen, presumably due to loss of dye, that occurs in experiments of longer duration.

Reversal of the niguldipine-induced Ca^{2+} increase could be achieved by addition of BSA, which resulted in a decrease in quin-2 fluorescence (Fig. 5). In the presence of more than 0.2% (w/v) BSA, $[Ca^{2+}]_i$ returned close to that of resting cells (Fig. 6). There is no linear relationship between the quin-2-derived fluorescence signal, that is shown in Fig. 5, and the corresponding cytoplasmic free Ca^{2+} concentration. Hence, for the sake of clarity, the BSA provoked decrease of $[Ca^{2+}]_i$ is shown as linear and semilogarithmic plots in Fig. 7. The 100% value refers to the Ca^{2+} concentration induced by B844-39 before addition of BSA and 0% refers to the Ca^{2+} concentration of 100 nM, obtained from other experiments with resting cells. Except for the first 2 min, the decrease appears to be of first order kinetics, since the semilogarithmic plot is linear (see insert of Fig. 7).

For comparison other substances were tested, which had common properties with niguldipine: cloxacepride [9], trifluoperazine and felodipine are as well calmodulin antagonists; nitrendipine, felodipine and the Ca^{2+} channel activator BAY K 8644 as DHP

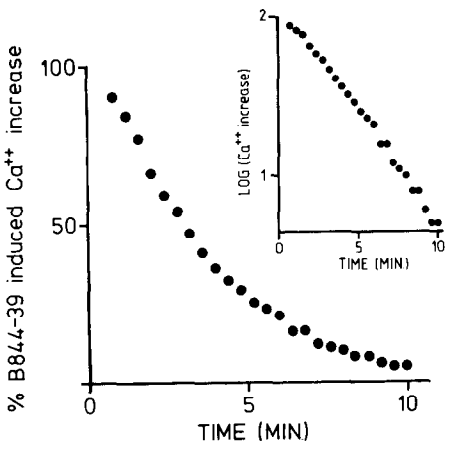

Fig. 7. Time-dependent decrease of cytoplasmic free Ca^{2+} after addition of 0.2% (w/v) BSA. BSA was added after stimulation with niguldipine as shown in Fig. 5. Cytoplasmic free Ca^{2+} before addition of BSA was taken as 100% and 0% refers to 100 nM as measured in unstimulated cells in other experiments. The symbols are mean values of 5 preparations.

derivatives are structurally related to niguldipine. In contrast to niguldipine, none of them increased cytoplasmic free Ca^{2+}, when tested at a concentration of 1 μM.

The effect of Ca^{2+} channel activators like CGP 28329 can be competitively antagonized by nitrendipine [3]. Therefore we simultaneously incubated cells with 4 μM nitrendipine and 500 nM B844-39. Under these conditions, nitrendipine did not modify the niguldipine-induced increase in comparison to cells treated only with B844-39.

Also ouabain and the α-adrenoceptor agonist phenylephrine (both 1 μM) did not mimic the effect of niguldipine.

DISCUSSION

The experiments reported in the present study show that racemic niguldipine at concentrations above 500 nM was able to substantially increase cytoplasmic free Ca^{2+} in mouse thymocytes.

This effect is stereospecific, since (−)-niguldipine is more active in increasing $[Ca^{2+}]_i$ than its (+)-enantiomer. In the case of different affinities for the molecular target of niguldipine, the same maximal response should be expected for racemic niguldipine and its enantiomers, but at higher concentrations for racemic and (+)-niguldipine. However, the three substances differ in their maximal response, which is produced in the same concentration range, indicating different efficacies.

The results obtained show that niguldipine raises $[Ca^{2+}]_i$ by initiating Ca^{2+} influx across the plasma membrane, since the effect is strictly dependent on extracellular Ca^{2+}. Of course, DPH derivatives that increase intracellular Ca^{2+} concentration might be Ca^{2+} channel activators. Indeed, a comparable increase of $[Ca^{2+}]_i$ was observed by other investigators with the Ca^{2+} channel activator CGP 28392 [3]. In human platelets this substance raised cytoplasmic Ca^{2+} to approximately 200 nM when applied in micromolar concentrations. The effect of CGP 28392 on platelets could be antagonized by nanomolar concentrations of nitrendipine. However, in our experiments with thymocytes, even 4 μM nitrendipine did not alter the effect elicited by 500 nM niguldipine as would be expected if niguldipine acted via the 1,4-DPH receptor site like CGP 28392. Furthermore, the Ca^{2+} channel activator BAY K 8644, also a DHP derivative, did not increase $[Ca^{2+}]_i$ in mouse thymocytes. Our experiments led us to the conclusion that niguldipine induces Ca^{2+} entry into thymocytes probably by a mechanism independent of the common DPH binding site.

The action of niguldipine as an agonist of Ca^{2+} entry into cells is an intriguing new aspect of this substance, since recent *in vivo* and *in vitro* studies indicate that to the contrary niguldipine acts as a potent Ca^{2+} channel blocker [5, 6]. In the report by Boer *et al.* [6] the binding characteristics of niguldipine to the 1,4-DPH binding site of L-type Ca^{2+} channels were studied in detail in guinea-pig skeletal muscle, heart muscle and brain membrane preparations. In their experiments they found a 40-fold higher affinity for the 1,4-DPH binding site with (+)-niguldipine as opposed to (−)-niguldipine. In contrast, the increase of cytoplasmic free Ca^{2+} in mouse thymocytes is induced more effectively by (−)-niguldipine as compared with the (+)-enantiomer. Moreover, this is due to differences in efficacy and not due to different binding affinities as in case of the typical DHP binding site. This finding further strengthens the view that the effect of niguldipine on thymocytes depends on quite different molecular events as compared with its actions on other tissues. The fact that other DPH derivatives (e.g. felodipine and nitrendipine), which act similarly on skeletal muscle and heart muscle as niguldipine, failed in mimicking the effect of niguldipine on thymocytes underlines the assumption that this action may be unique for niguldipine.

To further substantiate our view of the stimulating effect of niguldipine on Ca^{2+} entry into thymocytes, we tested several agents that are known for their (potential) ability to increase the intracellular Ca^{2+} concentration. The α-adrenoceptor agonist phenylephrine, that increases $[Ca^{2+}]_i$ in liver cells at 1 μM via the generation of inositol 1,4,5-triphosphate [10] failed to mimic the effect of niguldipine on thymocytes.

Since niguldipine exhibits calmodulin antagonistic properties also (Gietzen *et al.*, unpublished data), we tested in addition several calmodulin antagonists (e.g. trifluoperazine, felodipine and cloxacepride) on the same cellular system. Calmodulin antagonists, which inhibit various enzyme systems in addition to the Ca^{2+}-transporting ATPase of plasma membranes, could lead to an elevated $[Ca^{2+}]_i$ if there were no mechanisms for compensation. However, none of the calmodulin antagonists tested were able to increase $[Ca^{2+}]_i$. In conclusion, none of the considered mechanisms, besides Ca^{2+} entry, may be responsible for the effect of niguldipine on mouse thymocytes.

So far, the results of our experiments do not specify

the molecular mechanism of Ca^{2+} entry, evoked by niguldipine. One possibility would be influx through cell specific Ca^{2+} channels. But also involvement of an ion antiporter could be taken into consideration, since indeed, DHP derivatives were found to affect ion exchangers localized in plasma membranes as well as in intracellular membranes (reviewed in Ref. 11). Inhibition of the Na^+/K^+-ATPase, which occurs even below 1 μM ouabain in lymphocytes [12], decreases the transmembrane Na^+ gradient and thus might indirectly reduce Ca^{2+} extrusion via the Na^+/Ca^{2+}-antiporter. But probably, this is compensated by other mechanisms, since 1 μM ouabain did not alter resting $[Ca^{2+}]_i$ in mouse thymocytes, hence, excluding an explanation of the niguldipine effect by especially this ion exchange system.

Niguldipine is a highly lipophilic agent (log P = 4.3; octanol/H_2O at pH 7.4) [5] and therefore accumulates in lipophilic compartments of the cell such as the plasma membrane. When starting our experiments with racemic niguldipine, we wondered whether the drug increases intracellular Ca^{2+} concentration by an unspecific membrane disrupting effect. In this case, increased permeability not only for Ca^{2+} but also for other ions would occur. However, this possibility could be ruled out for niguldipine in the investigated concentration range as checked by the Mn^{2+} method described above. In contrast to the action of niguldipine, the Ca^{2+} ionophores A23187 or ionomycin and even more membrane permeabilizing agents like Triton X-100 (see Fig. 4) are less selective in that they allow also Mn^{2+} entry [14]. Moreover, in platelets, neutrophils and endothelial cells, even physiological stimuli, that induce Ca^{2+} influx from the extracellular space, in addition elicit Mn^{2+} entry which could be detected by a marked drop in intracellular quin-2 or fura-2 fluorescence intensity [13–16]. Interestingly other cell types discriminate between Ca^{2+} and Mn^{2+} influx, as shown for carbachol-stimulated rat parotid acinar cells [14] and Con A-stimulated mouse thymocytes [17] both responding solely with Ca^{2+} entry. Taken together, these facts indicate that niguldipine may induce Ca^{2+} influx into thymocytes by interference with a specific mechanism that may be related, at least in part, to that mechanism activated by Con A or to that described for carbachol-stimulated parotid cells [14].

Another interesting aspect is the reversal of the niguldipine-induced Ca^{2+} increase by the addition of BSA. Presumably, niguldipine is removed from the thymocytes by unspecific binding to BSA, comparable to the plasma protein binding as known from many drugs. This shows that the cellular mechanisms responsible for the reset of the cytosolic Ca^{2+} concentration of resting cells, extrusion and/or sequestration, were not impaired during the action of niguldipine. Since protein concentrations of body fluids are even higher than in our *in vitro* experiments, elevation of cytoplasmic free Ca^{2+} of thymocytes may be absent under *in vivo* conditions. Anyhow, niguldipine may provide an interesting tool to devise research in cellular biology. Further investigations to clarify the mode of action could help to unravel the molecular aspects of Ca^{2+} entry into thymocytes.

REFERENCES

1. Godfraind T, Miller R and Wibo M, Calcium antagonism and calcium entry blockade. *Pharmacol Rev* **38**: 321–416, 1986.
2. Godfraind T, Classification of calcium antagonists. *Am J Cardiol* **59**: 11B–23B, 1987.
3. Erne P, Buergisser E, Buehler FR, Dubach B, Kuehnis H, Meier M and Rogg H, Enhancement of calcium influx in human platelets by CGP 28 392, a novel dihydropyridine. *Biochem Biophys Res Commun* **118**: 842–847, 1984.
4. Schramm M, Thomas G, Towart R and Franckowiak G, Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature* **303**: 535–537, 1983.
5. Sanders KH, Kolassa N and Cettier B, B844-39, A new dihydropyridine derivative with slow onset and long duration of hypotensive action in comparison with nitrendipine. *Ann NY Acad Sci* **522**: 531–532, 1988.
6. Boer R, Grassegger A, Schudt C and Glossmann H, (+)-Niguldipine binds with very high affinity to Ca^{2+} channels and to a subtype of α-adrenoceptors. *Eur J Pharmacol* **172**: 131–146, 1989.
7. Hallam TJ, Sanchez A and Rink TJ, Stimulus response coupling in human platelets. *Biochem J* **218**: 819–827, 1984.
8. Cobbold PH and Rink TJ, Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem J* **248**: 313–328, 1987.
9. Gigl G, Hartweg D, Sanchez-Delgado E, Metz G, and Gietzen K, Calmodulin antagonism: a pharmacological approach for the inhibition of mediator release from mast cells. *Cell Calcium* **8**: 327–344, 1987.
10. Charest R, Blackmore PF, Berthon B and Exton JH, Changes in free cytosolic Ca^{2+} in hepatocytes following α-adrenergic stimulation. *J Biol Chem* **258**: 8769–8773, 1983.
11. Zernig G, Widening potential for Ca^{2+} antagonists: non-L-type Ca^{2+} channel interaction. *Trends Pharmacol Sci* **11**: 38–44, 1990.
12. Zimlichman R, Goldstein DS, Zimlichman S, and Keister HR, Effects of ouabain on cytosolic calcium in lymphocytes, platelets and adrenomedullary cells. *J Hypertens* **5**: 605–609, 1987.
13. Hallam TJ and Rink TJ, Agonists stimulate divalent cation channels in the plasma membrane of human platelets. *FEBS Lett* **186**: 175–179, 1985.
14. Merritt JE and Hallam TJ, Platelets and parotid ancinar cells have different mechanisms for agonist stimulated divalent cation entry. *J Biol Chem* **263**: 6161–6164, 1988.
15. Andersson T, Dahlgren C, Pozzan T, Stendahl O and Lew PD, Characterization of fMet-Leu-Phe receptor mediated Ca^{2+} influx across the membrane of human neutrophils. *Mol Pharmacol* **30**: 437–443, 1986.
16. Hallam TJ, Jacob R and Merritt JE, Evidence that agonists stimulate bivalent-cation influx into human endothelial cells. *Biochem J* **255**: 179–184, 1988.
17. Hesketh RT, Smith GA, Moore JP, Taylor MV and Metcalfe JC, Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. *J Biol Chem* **258**: 4876–4882, 1983.