Biochemical Pharmacology, Vol. 38, No. 24, pp. 4461–4465, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INHIBITORY ACTION OF CARVEDILOL, A NOVEL α,β-ADRENOCEPTOR ANTAGONIST, ON CATECHOLAMINE SECRETION AND CALCIUM INFLUX IN CULTURED BOVINE ADRENAL CHROMAFFIN CELLS

KYOJI MORITA,* SHUICHI HAMANO, MASANORI YOSHIZUMI and MOTOO OKA
Department of Pharmacology, Tokushima University School of Medicine, Tokushima 770, Japan

(*Received* 7 *March* 1989; *accepted* 26 *July* 1989)

Abstract—The effect of carvedilol on the secretory function was studied using cultured bovine adrenal chromaffin cells. Carvedilol caused the concentration-dependent inhibition of catecholamine secretion evoked by carbamylcholine, high K^+ or veratridine. The drug also caused the inhibition of radioactive calcium uptake stimulated by these secretagogues into the cells, and the inhibition of calcium uptake was observed in parallel with that of catecholamine secretion. The inhibitory action of carvedilol on catecholamine secretion was shown to be similar to that caused by a classical β-adrenoceptor antagonist, propranolol. Furthermore, although the level of carbamylcholine-stimulated catecholamine secretion inhibited by diltiazem, a potent calcium channel antagonist, was significantly raised by elevating the calcium concentration in the reaction mixture, increasing the concentration of calcium ions in the mixture failed to induce any substantial influence on the secretion inhibited by carvedilol, as well as propranolol, under the same experimental conditions. These results seem to indicate that carvedilol may cause the inhibition of catecholamine secretion through its blocking action on calcium influx into the cells, and suggest the possibility that the inhibitory action of carvedilol on calcium influx is presumably based on its stabilizing action on the plasma membranes rather than its blocking action on the calcium channels in the chromaffin cell.

Carvedilol has been developed as a β-adrenoceptor antagonist, and reported to be effective in the treatment of essential hypertension [1–3] and exercise-induced ischemic heart disease [4]. In addition to the β-adrenergic blocking action, carvedilol has also been reported to have a peripheral vasodilating action [5, 6], but the mechanism of this vasodilating action has not been completely elucidated. As a possible mechanism of the vasodilating action of carvedilol, the inhibitory action of this drug on α_1-adrenoceptors has previously been proposed [7, 8]. In addition, study of the effects of various antihypertensive drugs on the pressor response to different-type agonists in spontaneously hypertensive rats has provided further evidence confirming that the vasodilating action of carvedilol is probably due to its blocking action on the post-junctional α_1-adrenoceptor [9]. On the other hand, since the contraction of vascular vessels induced by a calcium channel activator, Bay K 8644, or 4-aminopyridine has recently been reported to be inhibited by carvedilol in a similar manner to calcium channel antagonists, carvedilol has therefore been suggested to produce a vasodilation probably through its blocking action on the voltage-dependent calcium channels in the blood vessels [10]. However, it still remains to be elucidated whether the vasodilating action of carvedilol is due to its inhibitory action on calcium influx into vascular smooth muscle cells.

A critical role of calcium ions in the secretory mechanisms of various neurotransmitters and hormones has already been proposed. It has been established that catecholamine secretion is initiated by a rise in the intracellular concentration of free calcium ions [11] resulting from the influx of extracellular calcium ions into the cell [12–14]. Catecholamine secretion from adrenal chromaffin cells is therefore considered to be a suitable index to reflect a possible alteration in the activity of the calcium transport process. To answer the question of whether carvedilol may cause a blocking action on the voltage-dependent calcium channels, we examined the effect of this drug on catecholamine secretion and calcium influx using cultured bovine adrenal chromaffin cells.

MATERIALS AND METHODS

Cell preparation and culture. Chromaffin cells were enzymatically prepared from fresh bovine adrenal glands according to the method described previously [15]. Briefly, the medulla was sliced with a hand slicer instead of cut into fine pieces, and the slices were then digested in the medium containing 0.1% collagenase, 0.01% soybean trypsin inhibitor, and 0.5% bovine serum albumin in balanced salt solution [135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/NaOH, pH 7.4]. Cells were plated on to 24-well cluster plates at a density of 5×10^5 cells/well, and maintained for 3 days as monolayer cultures in 1.5 ml of Eagle's minimum essential medium containing 5% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), gentamicin (40 μg/ml), fungizone (2.5 μg/ml) and 10 μM cytosine arabinoside.

* To whom correspondence should be addressed.

Determination of catecholamine secretion. Plated cells were washed with 1 ml of balanced salt solution, and then incubated at 37° for 10 min in 250 μl of balanced salt solution containing the drugs and/or different secretagogues. At the end of the incubation period, the medium was removed and the cells were lysed by adding 250 μl of 10% acetic acid and by subjecting a freeze–thaw cycle. Both the medium and the cell lysate were centrifuged in an Eppendorf centrifuge at the maximum speed (approx. 8800 *g*) for 2 min, and the supernatant fractions were then saved for catecholamine assay. Catecholamines were measured according to the method described previously [16]. Catecholamine secretion was expressed as percentage of the total cellular content secreted during the incubation period.

Measurement of calcium uptake. Plated cells were incubated with the drug and/or secretagogues at 37° for 10 min in balanced salt solution containing $^{45}CaCl_2$ (3 μCi/ml). At the end of the incubation period, the medium covering the cells was discarded and the cells were washed four times with 1 ml of ice-cold calcium-free balanced salt solution. These cells were solubilized by 1% Triton X-100, and the radioactivity in the lysate was determined with a liquid scintillation counter. The amount of calcium taken up into the cells was calculated on the basis of the specific activity of radioactive calcium ions in the reaction mixture.

The concentration of Na^+ in high K^+ medium was reduced to keep the solution isotonic. The Ca^{2+} concentration in high Ca^{2+} medium was 8 mM. The final concentration of dimethylsulfoxide, which was used to dissolve the hydrophobic drugs, in the incubation mixture was less than 1% which did not affect catecholamine secretion.

Chemicals. Carvedilol was kindly donated from Daiichi Seiyaku Co. (Tokyo, Japan). $^{45}CaCl_2$ was purchased from Amersham (Tokyo, Japan). Diltiazem and propranolol were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Carbamylcholine, veratridine, and A23187 were obtained from Sigma Chemical Co. (St Louis, MO). Other chemicals used were of commercially available reagent grade.

RESULTS

The effect of carvedilol on the secretory response of cultured bovine adrenal chromaffin cells was examined to indicate a possible effect of this drug on the calcium-related functions of these cells. As shown in Fig. 1, carvedilol inhibited catecholamine secretion evoked by 100 μM carbamylcholine in a manner dependent on its concentration. The significant inhibitory action of this drug on the secretory response to carbamylcholine was observed at concentrations higher than 10^{-5} M, and a maximum inhibition was obtained at 10^{-4} M. Carvedilol did not cause any significant change in the basal level of the secretion at any concentration used. In addition, catecholamine secretion evoked by depolarization of the plasma membranes was inhibited by carvedilol (Fig. 2). Carvedilol caused a significant inhibitory action on the secretion evoked by the depolarizing agents, high K^+ (56 mM) and veratridine (20 μM).

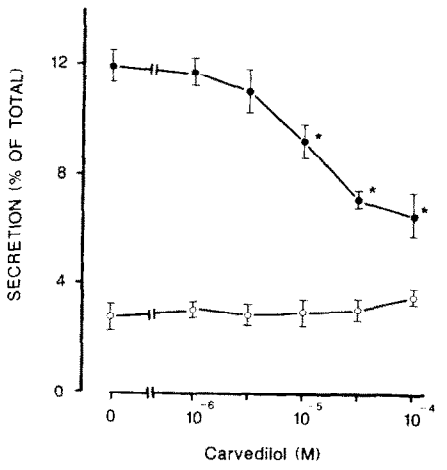

Fig. 1. Effect of carvedilol on catecholamine secretion evoked by carbamylcholine from cultured bovine adrenal chromaffin cells. Cells were incubated with (●) or without (○) 100 μM carbamylcholine at 37° for 10 min in the presence of various concentrations of carvedilol. Catecholamine secretion was determined as described in the text. Values are the mean ± SD of four experiments. *$P < 0.005$.

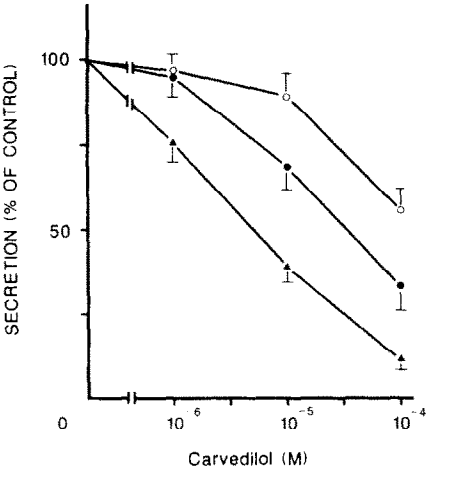

Fig. 2. Effect of carvedilol on catecholamine secretion evoked by different secretagogues from cultured bovine adrenal chromaffin cells. Cells were incubated with 56 mM KCl (○), 100 μM carbamylcholine (●), or 20 μM veratridine (▲) at 37° for 10 min in the presence of various concentrations of carvedilol. Catecholamine secretion was determined as described in the text. Results were expressed as per cent of the control value, which was calculated as 12.6 ± 0.5, 9.2 ± 0.9, 11.3 ± 0.4% of the total cellular content for the high K^+-, carbamylcholine-, and veratridine-induced secretion, respectively. Values are the mean ± SD of three experiments.

As compared with the effect of carvedilol on the secretion evoked by carbamylcholine, the inhibitory action of this drug on the secretion evoked by veratridine was observed to be more pronounced, and that on the secretion evoked by high K^+ was less pronounced. In contrast, the secretion evoked by a calcium ionophore, A23187, was not significantly influenced by carvedilol (Fig. 3). Furthermore, the effect of carvedilol on the accumulation of radioactive calcium ions within the cells was examined to

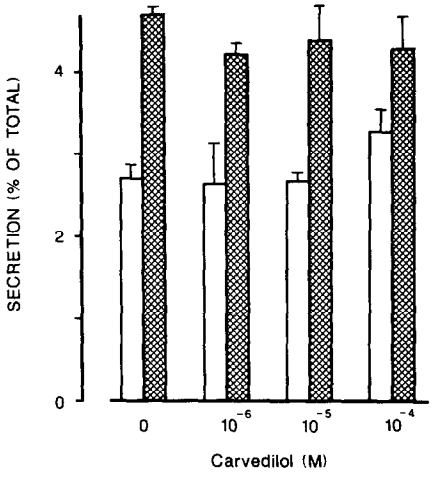

Fig. 3. Effect of carvedilol on catecholamine secretion evoked by A23187 from cultured bovine adrenal chromaffin cells. Cells were incubated with (hatched column) or without (open column) 10 μM A23187 at 37° for 10 min in the presence of various concentrations of carvedilol. Catecholamine secretion was determined as described in the text. Values are the mean ± SD of three experiments.

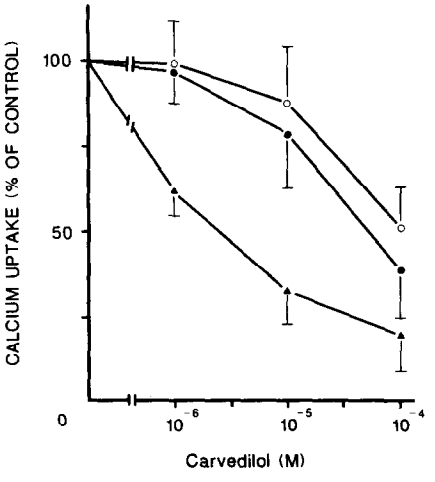

Fig. 4. Effect of carvedilol on radioactive calcium uptake stimulated by different secretagogues into cultured adrenal chromaffin cells. Cells were incubated with 56 mM KCl (○), 100 μM carbamylcholine (●), or 20 μM veratridine (▲) at 37° for 10 min in the incubation medium containing various concentrations of carvedilol and $^{45}CaCl_2$ (3 μCi/ml). Radioactive calcium uptake was determined as described in the text, and the results were then expressed as per cent of the control, which was calculated as 2.92 ± 0.22, 1.03 ± 0.08, 1.69 ± 0.09 nmol/10^6 cells for the uptake stimulated by high K^+, carbamylcholine, and veratridine, respectively. Values are the mean ± SD of three experiments.

elucidate a possible relationship between the effect on calcium transport and the inhibition of catecholamine secretion observed here. As shown in Fig. 4, carvedilol caused an inhibitory action on calcium uptake stimulated by carbamylcholine or depolarizing agents in an almost similar manner to its inhibitory action on the secretion.

The effect of propranolol on catecholamine secretion evoked by carbamylcholine was studied to

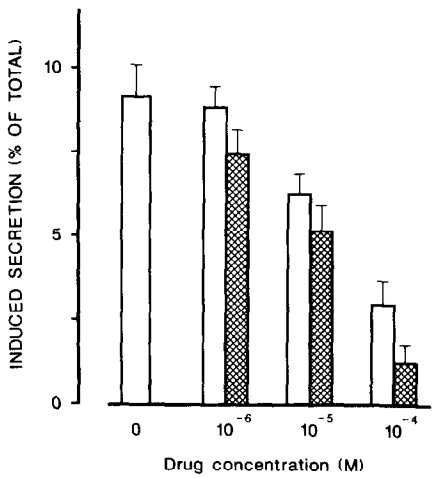

Fig. 5. Effects of carvedilol and propranolol on carbamylcholine-induced catecholamine secretion from cultured adrenal chromaffin cells. Cells were stimulated by 100 μM carbamylcholine at 37° for 10 min in the presence of different concentrations of carvedilol (open column) or *d,l*-propranolol (hatched column). The secretion was determined as described in the text. Results were expressed as the induced secretion, which was calculated by subtracting the value obtained in the absence of carbamylcholine from that obtained in the presence of the secretagogue. Values are the mean ± SD of three experiments.

compare with that of carvedilol on the secretory response. The carbamylcholine-induced secretion was markedly inhibited by propranolol, and this inhibitory action was observed to be nearly identical to that of carvedilol at the concentration range used (Fig. 5). Furthermore, to elucidate whether the inhibitory action of carvedilol on catecholamine secretion is attributed to its ability to act as a calcium channel antagonist, the inhibitory effects of carvedilol, propranolol, and diltiazem on the secretory response in the presence of a high concentration of calcium ions were compared. As shown in Fig. 6, catecholamine secretion evoked by carbamylcholine was enhanced by elevating the calcium concentration in the incubation medium from 2 mM to 8 mM. Under these conditions, the carbamylcholine-induced secretion was inhibited by carvedilol, propranolol, and diltiazem in the normal medium, and the secretion inhibited by carvedilol or propranolol was not significantly affected by elevating the calcium concentration. In contrast, the secretion inhibited by diltiazem was elevated by increasing the calcium concentration in the incubation medium. Thus, the inhibitory action of carvedilol on catecholamine secretion evoked by carbamylcholine was shown to be similar to that of propranolol, but different from that of diltiazem. In addition, the secretory response to high K^+ was also found to be significantly inhibited by carvedilol, propranolol, or diltiazem, and the inhibited secretion was furthermore shown to be elevated by increasing the calcium concentration in the incubation medium (Fig. 7).

DISCUSSION

The effect of carvedilol on the secretory response

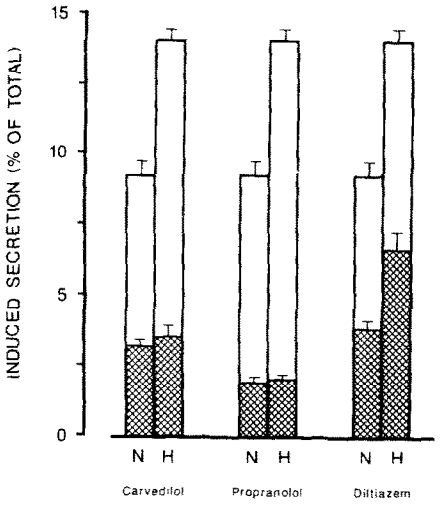

Fig. 6. Influence of high calcium concentration on the inhibition of carbamylcholine-induced catecholamine secretion by carvedilol, propranolol, and diltiazem in cultured adrenal chromaffin cells. Cells were incubated with (hatched column) or without (open column) 100 μM carvediol, 100 μM propranolol, or 10 μM diltiazem at 37° for 10 min in normal (N) and high Ca^{2+} medium (H) containing 100 μM carbamylcholine. The secretion was determined as described in the text, and the results were expressed as the induced secretion, which was calculated by subtracting the value obtained in the absence of carbamylcholine from that obtained in the presence of the secratagogue. Values are the mean ± SD of three experiments.

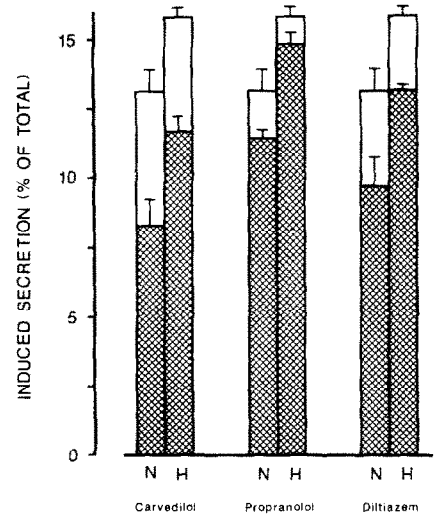

Fig. 7. Influence of high calcium concentration on the inhibition of high K^+-induced catecholamine secretion by carvedilol, propranolol, and diltiazem in cultured adrenal chromaffin cells. Cells were incubated with (hatched column) or without (open column) 100 μM carvedilol, 100 μM propranolol, or 10 μM diltiazem at 37° for 10 min in the high K^+-medium containing normal (N) or high concentration (H) of Ca^{2+}. The secretion was determined as described in the text, and the results were expressed as the induced secretion, which was calculated by subtracting the basal secretion from the stimulated secretion. Values are the mean ± SD of three experiments.

of cultured bovine adrenal chromaffin cells was studied to indicate its inhibitory action on the calcium-dependent cell functions. Carvedilol was shown to cause an inhibitory action on the secretory response to carbamylcholine (Fig. 1) or the depolarizing agents, high K^+ and veratridine (Fig. 2). In contrast, carvedilol failed to cause any significant effect on the secretion evoked by a calcium ionophore, A23187, which is considered to cause the activation of the intracellular secretory system as a result of introducing calcium ions directly into the cell interior (Fig. 3). These results indicate that the inhibitory action of carvedilol on catecholamine secretion can probably be attributed to its actions on the cell surface, presumably at sites other than acetylcholine receptors. Carvedilol may cause its inhibitory action on secretion by blocking the transport of calcium ions through the plasma membranes rather than by disturbing the exocytotic mechanism within the cells. In fact, carvedilol caused an inhibitory action on the accumulation of radioactive calcium ions within chromaffin cells stimulated by activation of acetylcholine receptors and depolarization in parallel with the inhibition of catecholamine secretion (Figs 2 and 4). Carvedilol is therefore considered to inhibit the secretory response presumably through its blocking action on the calcium influx into the cells.

Earlier studies have shown that a classical β-adrenoceptor antagonist, propranolol, can inhibit catecholamine secretion from perfused adrenal glands, and this inhibitory action is attributed to its stabilizing action on the plasma membranes [17]. Recently, carvedilol has been reported to have a local anaesthetic action on sympathetic nerve bundles at high concentrations [8]. In view of these findings, it seems reasonable to presume that carvedilol may inhibit calcium influx into chromaffin cells through a stabilizing action on the plasma membranes, thus resulting in the inhibition of the secretory response. In the present study, the inhibitory action of carvedilol on catecholamine secretion evoked by carbamylcholine was shown to be similar to that of propranolol (Figs 5 and 6). In contrast, the inhibitory action of carvedilol on the secretory response was shown to be clearly different from the inhibitory action of a calcium channel antagonist, diltiazem (Fig. 6). Like the inhibitory actions of local anaesthetics on the secretory response to high K^+, which has previously been shown to be restored by elevating the calcium concentration [17], the high K^+-induced secretion inhibited by carvedilol was also found to be significantly restored by increasing the calcium concentration in the incubation medium (Fig. 7). These observations therefore seem to indicate that, like the effect of propranolol described in the earlier report, the inhibitory action of carvedilol on the secretory response of the adrenal chromaffin cell is presumably due to its nonspecific stabilizing action on the plasma membranes rather than its specific blocking action on the calcium channels.

In the present study, we have found that carvedilol causes the inhibition of catecholamine secretion as a result of blocking the transport of calcium ions into cultured bovine adrenal chromaffin cells. Although carvedilol has already been reported to cause a blocking action on the calcium channels is blood vessels,

we have shown that the inhibitory action of carvedilol on calcium influx into chromaffin cells may be attributed to its stabilizing action on the plasma membranes rather than its blocking action on the calcium channels. Furthermore, since the concentration of the drug required for inhibiting the secretory response is relatively higher than that required for blocking the adrenoceptors [8], it therefore seems reasonable to conclude that the inhibitory action of carvedilol on catecholamine secretion is probably not related to its therapeutic actions.

REFERENCES

1. Meyer-Sabellek WA, Schulte K-L, Thiede HM and Gotzen R, Acute hypotensive response to the new antihypertensive agent (BM 14,190) in essential hypertensive patients. *J Hypertens* **1**(Suppl. 2): 351–352, 1983.
2. Eggertsen R, Sivertsson R, Andren L and Hansson L, Haemodynamic effects of carvedilol, a new beta-adrenoceptor blocker and precapillary vasodilator in essential hypertension. *J Hypertens* **2**: 529–534, 1984.
3. Ogihara T, Ikeda M, Goto Y, Yoshinaga K, Kumahara Y, Ishii M, Murakami E and Takeda T, The effect of low dose carvedilol on circadian variation of blood pressure in patients with essential hypertension. *J Cardiovasc Pharmacol* **10**(Suppl. 11): S108–S112, 1987.
4. Kaski JC, Rodriguez-Plaza L, Brown J and Maseri A, Efficacy of carvedilol (BM 14,190), a new beta-blocking drug with vasodilating properties in exercise induced ischemia. *Am J Cardiol* **56**: 35–40, 1985.
5. Sponer G, Bartsch W, Strein K, Muller-Beckmann B and Bohm E, Pharmacological profile of carvedilol as a β-blocking agent with vasodilating and hypotensive properties. *J Cardiovasc Pharmacol* **9**: 317–327, 1987.
6. Tanaka M, Masumura H, Tanaka S and Akashi A, Studies on the antihypertensive properties of carvedilol, a compound with β-blocking and vasodilating effects. *J Cardiovasc Pharmacol* **10**(Suppl. 11): S52–S57, 1987.
7. Cubeddu LX, Fuenmayor N, Varin F, Villagra VG, Colindres RE and Powell JR, Clinical pharmacology of carvedilol in normal volunteers. *Clin Pharmacol Ther* **41**: 31–44, 1987.
8. Seki N, Nagao T, Komori K and Suzuki H, Alpha and beta adrenoceptor blocking action of carvedilol in the canine mesenteric artery and vein. *J Pharmacol Exp Ther* **246**: 1116–1122, 1988.
9. Hashimoto H, Tanaka M, Kanda K and Akashi A, Analysis of the mechanism underlying the vasodilator action of carvedilol in pithed spontaneously hypertensive rats. *Drugs* **36** (Suppl. 6): 31–36, 1988.
10. Hattori Y, Nakaya H, Endo M, Nakao Y and Kanno M, Vascular effects of carvedilol, a new β-adrenoceptor antagonist with vasodilating properties, in isolated canine coronary artery. *J Cardiovasc Pharmacol* **13**: 572–579, 1989.
11. Knight DE and Kesteven NT, Evoked transient intracellular free Ca^{2+} changes and secretion in isolated bovine adrenal medullary cells. *Proc R Soc Lond [Biol]* **218**: 177–199, 1983.
12. Douglas WW and Poisner AM, On the mode of action of acetylcholine in evoking adrenal medullary secretion: increased uptake of calcium during the secretory response. *J Physiol (Lond)* **162**: 385–392, 1962.
13. Kilpatrick DL, Slepetis RJ, Corcoran JJ and Kirshner N, Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J Neurochem* **38**: 427–435, 1982.
14. Holz RW, Senter RA and Frye RA, Relationship between Ca^{2+} uptake and catecholamine secretion in primary dissociated cultures of adrenal medulla. *J Neurochem* **39**: 635–646, 1982.
15. Morita K, Ishii S, Uda H and Oka M, Requirement of ATP for exocytotic release of catecholamines from digitonin-permeabilized adrenal chromaffin cells. *J Neurochem* **50**: 644–648, 1988.
16. Morita K, Brocklehurst KW, Tomares SM and Pollard HB, The phorbol ester TPA enhances A23187-, but not carbachol- and high K^+-induced catecholamine secretion from cultured bovine adrenal chromaffin cells. *Biochem Biophys Res Commun* **129**: 511–516, 1985.
17. Jaanus SD, Miele E and Rubin RP, The analysis of the inhibitory effect of local anaesthetics and propranolol on adrenomedullary secretion evoked by calcium or acetylcholine. *Brit J Pharmacol Chemother* **31**: 319–330, 1967.