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ω-Conotoxin binding and effects on calcium channel function in human neuroblastoma and rat pheochromocytoma cell lines

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Binding of ω-conotoxin, a peptide toxin specific for some subtypes of voltage-operated calcium channels (VOCCs), was investigated in IMR32 neuroblastoma and PC12 pheochromocytoma cell lines. In both cell types, binding was specific, saturable and of high affinity. Association was rapid and dissociation almost non-existent. Dihydropyridines and verapamil failed to affect toxin binding, while high concentrations of $CaCl_2$ completely antagonized it. Depolarization with high K^+ induced a $[Ca^{2+}]_i$ rise (revealed by the fura-2 fluorimetric technique) that consisted of an initial (0.5–1 min) peak followed by a prolonged (several minutes) plateau phase. ω-Conotoxin blocked mainly the first phase, while the dihydropyridine Ca^{2+} channel blocker, nitrendipine, primarily affected the plateau. This result suggests that in the two cell lines investigated, ω-conotoxin acts mainly on a subgroup of VOCCs that is resistant to dihydropyridines.

ω-Conotoxin; Ca^{2+} channel; (Neuroblastoma, Pheochromocytoma)

1. INTRODUCTION

Peptide neurotoxins have been fundamental tools for the study of membrane receptors and channels of excitable cells, such as the nicotinic receptor and Na^+ and K^+ channels. More recently, toxins that interact with voltage-operated Ca^{2+} channels (VOCCs) have been described [1]. Among these, ω-conotoxin (ωCTx) purified from the marine snail, *Conus geographus*, was shown to bind to and block VOCCs in a variety of neuronal preparations [2,6]. No binding or effect of ω-conotoxin (ωCTx) was detected in heart, skeletal or smooth muscle cells [4–6,7].

Up to now, biochemical investigation of the mechanisms of ωCTx action has been severely affected by the heterogeneity of the nervous tissue and of the preparations obtained therefrom (synaptosomes, dissociated neurons). A few cultured cell lines, on the other hand, are known to maintain essential properties of nerve cells, and therefore represent convenient tools for the biochemical and pharmacological characterization of the underlying molecules and physiological mechanisms. Here, two such lines, IMR32 human neuroblastoma and PC12 rat pheochromocytoma, have been employed to investigate the binding of ωCTx and its effects on the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$. The results obtained demonstrate, for the first time, the presence of ωCTx-binding sites in cultured cells and characterize in further detail the features of the VOCCs expressed in the two cell lines [8,9].

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Abbreviations: ^{125}I-ωCTx, iodinated ω-conotoxin (fraction VIA); $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; VOCCs, voltage-operated Ca^{2+} channels

2. MATERIALS AND METHODS

2.1. *Cell culture*

The source and culture conditions of IMR-32 cells have been

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described [8]. PC12 cells were grown in RPMI 1640 medium, supplemented with 10 μg/ml of insulin and transferrin, 6 mg/l putrescine, 62 μg/l progesterone, 40 μg/l selenite and 0.5 mg/ml bovine serum albumin.

2.2. *^{125}I-ωCTx binding*

IMR32 and PC12 cells were detached mechanically and then homogenized in a Potter glass homogenizer in buffer A (0.3 M sucrose, 5 mM Hepes/Tris, pH 7.4) containing 0.01 mg/ml lysozyme. Saturation experiments were performed in buffer A supplemented with 1 mg/ml bovine serum albumin (BSA) in Eppendorf tubes containing, in a total volume of 100 μl, 50–100 μg proteins and ^{125}I-ωCTx at $0.1\text{–}100 \times 10^{-12}$ M. To estimate non-specific binding parallel aliquots were preincubated with an excess (1.6×10^{-8} M) of unlabelled ωCTx. Mixture was incubated at 37°C for 1 h, then diluted with 1 ml ice-cold buffer B (160 mM choline chloride, 1.5 mM $CaCl_2$, 5 mM Hepes/Tris, pH 7, with 1 mg/ml BSA) and finally centrifuged for 3 min at $10\,000 \times g$. Supernatants were discarded, the pellets being rinsed once with ice-cold buffer B and then counted (Beckman, gamma 4000 counter). The effect of cold ωCTx, drugs and ions on the equilibrium binding of ligand was estimated as described above, but various substances were added to the membrane preparations 10 min before ^{125}I-ωCTx, which was used at a constant concentration of 1×10^{-11} M. This same concentration was used for studying the time course of toxin association with washing being performed after the indicated intervals. Non-specific binding for each time point was determined as described above. The time course for ^{125}I-ωCTx dissociation was studied after 120 min of equilibrium binding by adding to the tubes a 10-fold volume of buffer A also containing an excess of unlabelled ωCTx (1.6×10^{-7} M). At different times tubes were processed as above.

2.3. *$[Ca^{2+}]_i$ measurements with fura-2*

IMR-32 or PC12 cells were gently detached from the dish, washed once in a Krebs-Ringer-Hepes solution (KRH) containing (in mmol/l): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 6 glucose and 25 Hepes-NaOH (pH 7.4), and resuspended (10^7 cells/ml) in the same solution. Loading of cells with fura-2 was achieved by incubating the cell suspension with 5 μM fura-2 acetoxy methyl ester for 30 min at 37°C. Treatment with ωCTx (1–10 μM) was carried out during cell loading, after which the cells were finally resuspended (1.3×10^6/ml) in KRH. Fluorescence measurements (excitation and emission at 345 and 490 nm, respectively) were made in a spectrofluorimeter using a thermostatically controlled cell holder equipped for continuous stirring. Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed as in [10]. Results shown are representative of at least 4 different experiments.

2.4. *Materials*

MEM, RPMI, FCS and antibiotics were obtained from Flow Labs (UK) while Petri dishes were from Falcon (USA). ωCTx, fraction VIA, was obtained from Peninsula Labs (USA), while ^{125}I-ωCTx, fraction VIA (spec. act. ≈ 2000 Ci/mmol) was from Amersham (England). Nitrendipine and Bay-K-8644 were a kind gift from Professor Govoni of our Department. Fura-2 was from Galbiochem (Behring). All other chemicals were reagent grade and purchased from Merck (FRG).

3. RESULTS AND DISCUSSION

3.1. *Binding of ω-conotoxin to two neural cell lines*

Fig.1 shows the results of typical binding experiments carried out by the use of ≈ 50 μg protein of either IMR-32 and PC12 cells. In both cell lines, binding was of high affinity, specific, and saturable. Non-specific binding represented 30–50% of total binding at the highest concentrations of the radioligand used. Non-linear regression analysis of untransformed experimental data reveals that the K_d for ^{125}I-ωCTx binding was 4.7 ± 1 and 7.2 ± 1.5 pM for IMR-32 and PC12 cells, respectively (n = 3). Values in the same range were found in competition experiments with cold ωCTx, illustrated in fig.2 (IC_{50} values of 5 and 4 pM were determined for IMR32 and PC12, respectively). These values, when transformed according to the Cheng-Prusoff equation, resulted in K_d values of 2.13 and 2.6 pM, respectively. Also, in these experiments, no evidence of site multiplicity was observed. The corresponding B_{max} values were 10.7 ± 0.8 and 7.4 ± 2 fmol/mg protein. From these values the number of ωCTx sites/cell can be roughly calculated to be ≈ 6000 and ≈ 5000

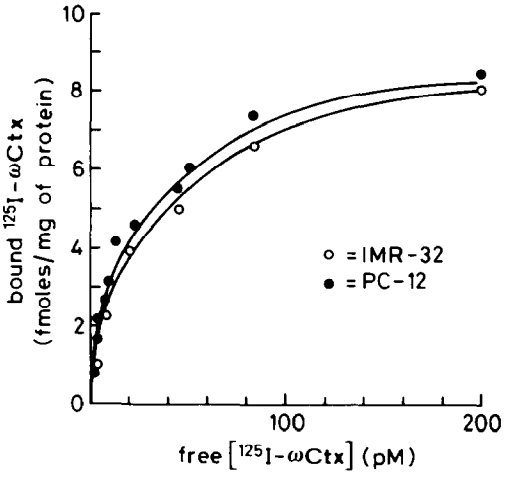

Fig.1. Equilibrium binding of ^{125}I-ωCTx to IMR32 and PC12 cell homogenates. Each value of this representative experiment is the mean of triplicate samples. Non-specific binding was measured as described. Non-linear regression analysis of different experiments gave the reported results for B_{max} (10.7 and 7.4 fmol/mg proteins for IMR32 and PC12 cells, respectively) and K_d (4.7 and 7.2×10^{-12} M for IMR32 and PC12 cells, respectively).

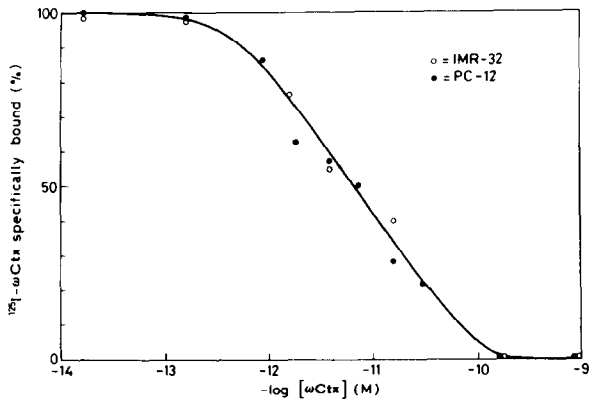

Fig.2. Inhibition of ^{125}I-ωCTx binding to IMR32 and PC12 cells by unlabelled ωCTx. The calculated IC_{50} values were 5 and 4×10^{-12} M (corresponding to K_d values of 2.13 and 2.6 pM) for IMR32 and PC12 cells, respectively.

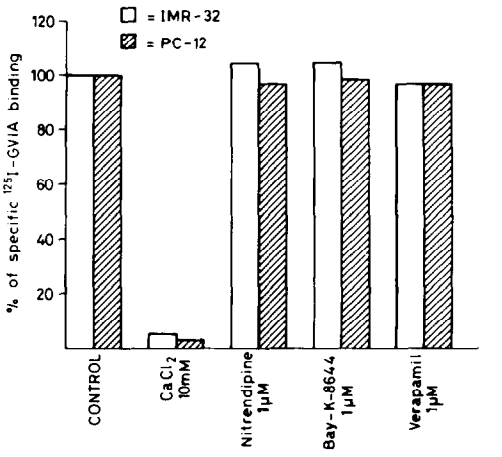

Fig.3. Inhibition of ^{125}I-ωCTx binding by classic Ca^{2+} channel antagonists and ions. These experiments were performed as described in the legend to fig.2 and section 2. Different experiments also gave similar results with higher (10 μM) drug concentrations (not shown).

for IMR32 and PC12, respectively. Fig.3 illustrates the effects of various VOCC ligands and ions on ^{125}I-ωCTx binding. Neither the antagonist dihydropyridine drug nitrendipine (lane C), nor the agonist Bay-K-8644 (lane D) (concentrations up to 10 μM) was able to affect significantly ^{125}I-ωCTx binding to IMR-32 and PC12 cells. Also, verapamil (which binds to a site of VOCCs different from that of the dihydropyridines), was ineffective (levels up to 10 μM). In different preparations, ωCTx binding, but not dissociation, was reported to be very sensitive to cations [4]. In our hands ^{125}I-ωCTx binding was also strongly inhibited (≈95 and ≈98% for IMR-32 and PC12 cells, respectively) in the presence of 10 mM $CaCl_2$ (lane B).

^{125}I-ωCTx specific binding was fast (reaching equilibrium in less than 15 min at 37°C) and essentially irreversible. In fact, no appreciable dissociation was observed in 10-fold diluted preparations that were incubated for up to 2 h with a large excess of unlabeled ωCTx (fig.4).

3.2. *Effects of CTx on K^+-induced $[Ca^{2+}]_i$ rises*

The effects of ωCTx and other treatments on $[Ca^{2+}]_i$ were investigated in both IMR-32 and PC12 cells using the Ca^{2+} indicator fura-2. Representative results from such experiments in PC12 cells are illustrated in fig.5. Similar results were obtained with IMR-32 cells as well (not shown). Fig.5a shows a typical trace obtained when a depolarizing concentration of KCl (50 mM) was added to PC12 cells. A very rapid $[Ca^{2+}]_i$ increase was detected, which was dependent upon the influx of calcium from the extracellular medium, since it was abolished in a Ca^{2+}-free medium containing 1 mM EGTA [8,9]. The K^+-induced $[Ca^{2+}]_i$ increase was biphasic, consisting of a fast, high initial peak (average 730 ± 50 nM, $n = 10$) reached after 10–12 s, followed by a prolonged plateau at much lower levels (200–400 nM). The dihydropyridine Ca^{2+} blocker drug, nitrendipine, used at concentrations up to 10 μM, was able to modify the initial $[Ca^{2+}]_i$ peak only in part (fig.5c), whereas it blocked the plateau completely (fig.5a,b). The effect of ωCTx on the K^+-induced $[Ca^{2+}]_i$ increase was completely different from that of nitrendipine. In fact, ωCTx (either preincubated for 30 min, or added to the cells after the initial, K^+-induced peak had subsided) had only a weak blocking effect on the plateau phase. In contrast, ωCTx strongly inhibited the initial rapid $[Ca^{2+}]_i$ peak induced by high K^+ (cf. fig.5b, peak 290 nM, with fig.5a, peak 670 nM) in both PC12 and IMR-32 cells (not shown). Moreover, when ωCTx and nitrendipine were added together before high K^+, both phases of the calcium increase were almost completely blocked (fig.5d).

The effect of ωCTx was highly specific on the

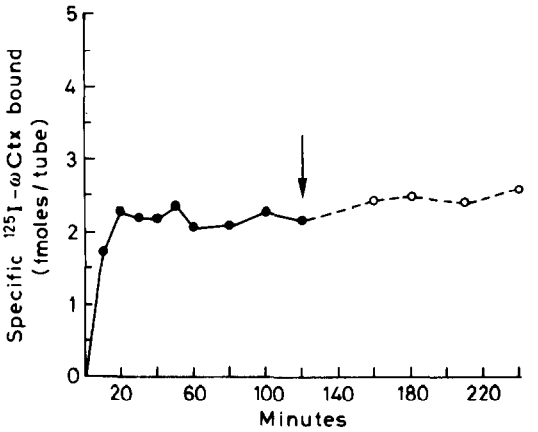

Fig.4. Association and dissociation kinetics of ^{125}I-ωCTx binding to PC12 cells. Each point represents only specific binding. Association was started by the addition of 1×10^{-11} M ^{125}I-ωCTx. After less than 15 min a plateau phase is reached. The addition of an excess of unlabelled toxin and sample dilution (arrow) did not dissociate the complex formed for more than 2 h at 37°C.

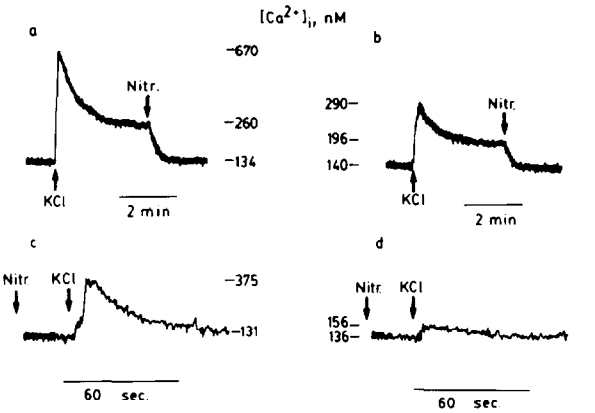

Fig.5. Effects of ωCTx and nitrendipine on the $[Ca^{2+}]_i$ rise induced by depolarization. In (a,b) it is shown that nitrendipine (1 μM) added after KCl blocks the opened calcium channels. When added before KCl (c) nitrendipine has no effects on the rapid and transient phase of calcium rise. ωCTx when added after KCl has no effects on the calcium channels blocked by nitrendipine (not shown) while when added before (b–d) it specifically blocks the rapid, nitrendipine-insensitive calcium channels. Note the different time scale (bars in the traces in c,d with respect to a,b), which allowed better resolution of the transient peaks.

VOCCs: both phases of the bradikinin-induced rise in $[Ca^{2+}]_i$ (the first related to the release of Ca^{2+} from internal stores and the second to the opening of receptor-operated, voltage-independent calcium channels) were completely unaffected by pretreatment of the cells with ωCTx (not shown).

Our data represent the first evidence that two continuous nerve cell lines, a human neuroblastoma and a rat pheochromocytoma, express high-affinity binding sites for ωCTx. The features of the binding interaction to the cell lines (K_d, insensitivity to Ca^{2+} channel blocker, inhibition by high Ca^{2+}) closely resemble those previously described in the nervous system and neuron-derived fractions [2–7]. Moreover, ωCTx markedly affected the $[Ca^{2+}]_i$ increase induced by K^+ depolarization via the activation of VOCCs. Thus, the two cell lines appear to be interesting tools for investigating the mechanism of action of ωCTx and the properties of VOCCs in these cultured cells. Previous electrophysiological studies in neurons [11,12] demonstrated that ωCTx blocks VOCCs of both the L (the typical dihydropyridine targets) and N type, while it spares the T type VOCCs. In muscle cells, on the other hand, L type channels are not affected by ωCTx. In the two cell lines we have now found that ωCTx blocked preferentially the initial $[Ca^{2+}]_i$ peak induced by K^+ depolarization, which was little affected by the dihydropyridine drug, nitrendipine. The latter, on the other hand, blocked preferentially the $[Ca^{2+}]_i$ plateau, which was apparently untouched by ωCTx. It is noteworthy that in PC12 cells, the first rapid peak of the calcium increase, that we have shown here to be blocked by ωCTx but not by nitrendipine, is actually correlated with the rapid phase of neurotransmitter secretion [9].

Although absolutely no kind of correlation can be drawn between these fluorimetric assays and the electrophysiological studies (since the cellular conditions and the recording of data are so different in the two cases), we can, however, suggest that Ca^{2+} channels of nerve cells can be, if possible, also more heterogeneous than had previously been believed. ωCTx blocks some 'rapid' channels in both IMR-32 and PC12 cells. Nitrendipine does not seem to block the same channels. On the other hand, we found in both neuronal cell lines a second population of channels (probably undetected and therefore not studied by electrophysiological means) which is very long lasting and practically not inactivating for tens of minutes. These channels, although present in nerve cells, can be block-

ed by dihydropyridines but not by ωCTx like the VOCCs present in muscle cells.

Further work is currently being performed to characterize better, using IMR32 and PC12 cells, the different 'subtypes' of neuronal calcium channels expressed and their relative functional role, before giving them any 'new' name. However, our data indicate that by combining such important tools as peptide neurotoxins and neuronal cell lines, we can really hope to gain much more information on the biochemical, pharmacological and functional properties of these channel molecules, known to be so important in the control of synaptic activity [13].

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