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SODIUM AND CALCIUM FLUXES IN A CLONAL NERVE CELL LINE

BY WILLIAM B. STALLCUP

From the Developmental Biology Laboratory, the Salk Institute, P.O. Box 1809, San Diego, California 92112, U.S.A.

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SUMMARY

1. $^{22}Na^+$ and $^{45}Ca^{2+}$ fluxes were studied in the clonal nerve cell line PC12. Three distinct types of ion channels were found: (*a*) voltage-dependent Na^+ channels, (*b*) voltage-dependent Ca^{2+} channels, and (*c*) acetylcholine-activated channels permeable to both ions.

2. $^{22}Na^+$ uptake through voltage-dependent Na^+ channels is induced by veratridine and scorpion venom, and is inhibited 50% by 5×10^{-7} M-tetrodotoxin and greater than 98% by 5×10^{-6} M-tetrodotoxin.

3. $^{45}Ca^{2+}$ uptake through voltage-dependent Ca^{2+} channels is induced by depolarizing the cells in 50 mM-KCl. This flux is not dependent on the presence of Na^+ in the medium and is insensitive to 5×10^{-6} M-tetrodotoxin. However, 1 mM-Mn^{2+} causes a 95% inhibition of K^+-induced $^{45}Ca^{2+}$ uptake.

4. Veratridine and scorpion venom also induce voltage-dependent $^{45}Ca^{2+}$ uptake which can be blocked by 1mM-Mn^{2+}. In contrast to KCl-induced $^{45}Ca^{2+}$ uptake, this flux is completely blocked by 5×10^{-6} M-tetrodotoxin and is abolished by removal of Na^+ from the medium. Thus the depolarizing stimulus for Ca^{2+} uptake in this case is Na^+ influx through voltage-dependent Na^+ channels.

5. Carbamylcholine induces both $^{22}Na^+$ and $^{45}Ca^{2+}$ fluxes which are blocked by nicotinic cholinergic antagonists with the exception of α-bungarotoxin. The $^{22}Na^+$ flux occurs exclusively via acetylcholine receptor channels, as evidenced by the lack of effect of 5×10^{-6} M-tetrodotoxin. In the presence of Na^+, almost all of the $^{45}Ca^{2+}$ uptake can be blocked by 1 mM-Mn^{2+} and thus occurs via voltage-dependent Ca^{2+} channels which are activated by the depolarizing Na^+ influx. 6–8% of the total $^{45}Ca^{2+}$ flux, however, is insenstitive to 1 mM-Mn^{2+}, suggesting that this portion of the uptake occurs via the acetylcholine receptor channels. In Na^+-free medium, the Mn^{2+}-resistant $^{45}Ca^{2+}$ component increases to 40% of the total uptake, apparently due to lack of competition from Na^+ for the acetylcholine receptor channels. This receptor-linked flux still causes sufficient depolarization to induce the additional 60% of the Ca^{2+} flux through voltage-dependent, Mn^{2+} sensitive Ca^{2+} channels.

6. Mn^{2+} inhibits Ca^{2+} flux through voltage-dependent Ca^{2+} channels by competing for entry through these channels. 50 mM-KCl induces $^{54}Mn^{2+}$ fluxes in PC12 cells that are comparable in magnitude to $^{45}Ca^{2+}$ fluxes.

7. In normal saline $^{45}Ca^{2+}$ efflux from PC12 cells is several times more rapid than in Na^+-free medium, indicating the presence of a Ca^{2+}-Na^+ exchange mechanism.

INTRODUCTION

Extracellular Ca^{2+} is known to be required for the release of neurotransmitters and hormones from a varitety of tissues (Douglas, 1968; Katz, 1969; Rubin, 1970). Adrenal chromaffin cells and sympathetic neurones, for example, provide well-studied cases in which the release of catecholamines and acetylcholine is dependent on the entry of Ca^{2+} into the cells. This Ca^{2+} entry is triggered by depolarization, suggesting the possible involvement of voltage-dependent Ca^{2+} channels in the excitation–secretion process. The necessary depolarization can be brought about by electrical stimulation (Furshpan, MacLeish, O'Lague, & Potter, 1976), by elevated concentrations of extracellular K^+ (Vogt, 1952; Douglas & Rubin, 1963; Boullin, 1967; Baker & Rink, 1975) and also by acetylcholine (Douglas & Poisner, 1962; Douglas, Kanno & Sampson, 1967*a*, *b*; Brandt, Hagiwara, Kidokoro & Miyazaki, 1976). Electrophysiological studies of cultured adrenal chromaffin cells (Brandt *et al.* 1976) indicate that Na^+ influx, either through the acetylcholine receptor channels or through voltage-dependent Na^+ channels which open in response to acetylcholine-mediated depolarization, could be important in triggering the subsequent increase in Ca^{2+} permeability and the resulting secretion of transmitter. Alternatively, sufficient Ca^{2+} might enter through the acetylcholine receptor channels to trigger secretion.

The clonal cell line PC12, derived from a rat pheochromocytoma (Greene & Tischler, 1976), appears to provide an excellent model for studying excitation–secretion in adrenal chromaffin cells and sympathetic neurones. The cells have receptors for acetylcholine (Ditcher, Tischler & Greene, 1977; Patrick & Stallcup, 1977*a*, *b*) and exhibit Na^+ action potentials (Ditchter *et al.* 1977). This cell line can also synthesize and store both catecholamines and acetylcholine (Greene & Tischler, 1976; Greene & Rein, 1977*b*; Schubert, Heinemann & Kidokoro, 1977) and moreover, can be induced to release these transmitters under depolarizing conditions (Greene & Rein, 1977*a*, *b*, *c*). Chalfie, Hoadley, Pastan & Perlman (1976) have demonstrated that these cells accumulate $^{45}Ca^{2+}$ under the same depolarizing conditions that trigger catecholamine release. We have now used the PC12 cell line to study in more detail the mechanisms by which Ca^{2+} entry occurs and the extent to which this Ca^{2+} entry may be dependent upon Na^+ flux as the primary depolarizing stimulus. The role of these fluxes in stimulus–secretion coupling is examined in detail in a parallel study of dopamine release from PC12 cells (Ritchie, 1979).

METHODS

Cell culture

The PC12 cell line was routinely grown in 100 mm Falcon tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% horse serum and 10% fetal calf serum (Grand Island Biological Co., New York, N.Y.). For ion flux assays, the cells were harvested and replated in fresh medium on polylysine-coated 35 mm tissue culture dishes. These cultures contained $1-2\times10^6$ cells each and were used within 2 days after plating.

Ion flux assays

$^{22}Na^+$, $^{45}Ca^{2+}$, and $^{54}Mn^{2+}$ were obtained from Amersham-Searle (Arlington Heights, Illinois). Ouabain (Sigma, St Louis, Missouri) was included in all ion flux assays at a concentration of 5 mM. Ouabain inhibits the Na^+-K^+ ATPase and allows the passive influx of Na^+ to be measured

in the absence of the competing process of active Na^+ extrusion. This enables us to make useful measurements of the kinetics of Na^+ uptake over an extended period of time (several minutes in the experiments described here). Ouabain was not allowed to preincubate with the cells before the assay, but was added at the beginning of the assay along with the isotope and effector of interest. Thus the ionic gradients were intact at the beginning of the assay. The background rate of uptake of $^{22}Na^+$ or $^{45}Ca^{2+}$ in the presence of ouabain alone was always small compared to the rates of uptake found in the presence of ouabain plus an effector such as carbamylcholine. The magnitudes of these background rates are given in Tables 1 and 2. In all cases the rates measured were initial rates of uptake. The background rates were subtracted from the rates observed in the presence of ouabain plus effectors to obtain the values presented in the figures and tables. These initial rates were normalized for the amount of cellular protein in the cultures, so that the final values are expressed in terms of moles min^{-1} mg^{-1}.

Flux assays using veratridine and venom from the scorpion *Tityus serrulatus* (Sigma) were performed at 37 °C as described previously (Catterall & Nirenberg, 1973; Stallcup & Cohn, 1976*a*, *b*; Stallcup, 1977). Veratridine works very poorly at lower temperatures. In cases where tetrodotoxin (Sankyo Chemical Co., Tokyo) was used it was not preincubated with the cells, but was added at the same time as the veratridine, scorpion venom and $^{22}Na^+$.

Fluxes stimulated by carbamylcholine chloride (Sigma) were measured as described by Patrick & Stallcup (1977*a*, *b*). 22 °C was chosen as the assay temperature because desensitization of the acetylcholine receptor (and hence more complex kinetics of ion flux) becomes more severe at 37 °C (Catterall, 1975*a*). When cholinergic antagonists were tested, they were allowed to preincubate with the cells prior to addition of the agonist and the $^{22}Na^+$ according to the following protocol: D-tubocurarine and hexamethonium (Sigma), 10 min at 22 °C; quinuclidinylbenzilate, 30 min at 22 °C; antibody to eel acetylcholine receptor (Patrick, Heinemann, Lindstrom, Schubert & Steinbach, 1972; Patrick & Lindstrom, 1973), 1 hr at 37 °C.

K^+-stimulated fluxes were initiated by including 50 mM-KCl in the assay medium (Stallcup & Cohn, 1976*a*). As detailed in the text, these measurements were carried out both at 22 and 37 °C for comparison with veratridine and carbamylcholine-induced fluxes.

Two assay solutions were routinely employed in these experiments. 'Normal' saline contained 130 mM-NaCl, 2 mM-$CaCl_2$, 5 mM-KCl, 5 mM-glucose, and 50 mM-hydroxy-ethyl-piperazine ethanesulphonic acid (HEPES) adjusted to pH 7·4. 'Na-free' saline was prepared by replacing the NaCl with 260 mM-sucrose.

RESULTS

Electrically excitable cells are known to have several types of ion channels that are involved in the generation and propagation of impulses. By inducing Na^+ and Ca^{2+} fluxes in PC12 with a variety of stimuli and by using specific inhibitors to dissect the responses into distinct components, we hoped to determine the relative contributions of various channels under the different conditions employed.

We anticipated that we might encounter two main types of ion channels in PC12.

(1) Voltage-dependent channels. Voltage-dependent Na^+ channels can be specifically blocked by tetrodotoxin (Evans, 1972) while voltage-dependent Ca^{2+} channels can be blocked by low concentrations of Mn^{2+} (Hagiwara, 1973).

(2) Ion channels associated with the acetylcholine receptor. Although there have been reports of agents that block these channels (for example, ceruleotoxin (Bon & Changeux, 1975)), as yet none of these is generally available. On the other hand, there are a variety of cholinergic antagonists such as D-tubocurarine which block the activation of the acetylcholine receptor.

Sodium uptake

In practice it is possible to measure $^{22}Na^+$ uptake in PC12 cells stimulated with carbamylcholine or with veratridine and scorpion venom in the absence of ouabain.

However, these fluxes are smaller, shorter in duration, and of a more complex nature than those observed in the presence of ouabain. This is to be expected, since in the absence of ouabain our measurements reflect the contributions of two competing processes, passive influx and active extrusion. Thus the use of ouabain is a convenience that improves our ability to measure Na^+ fluxes.

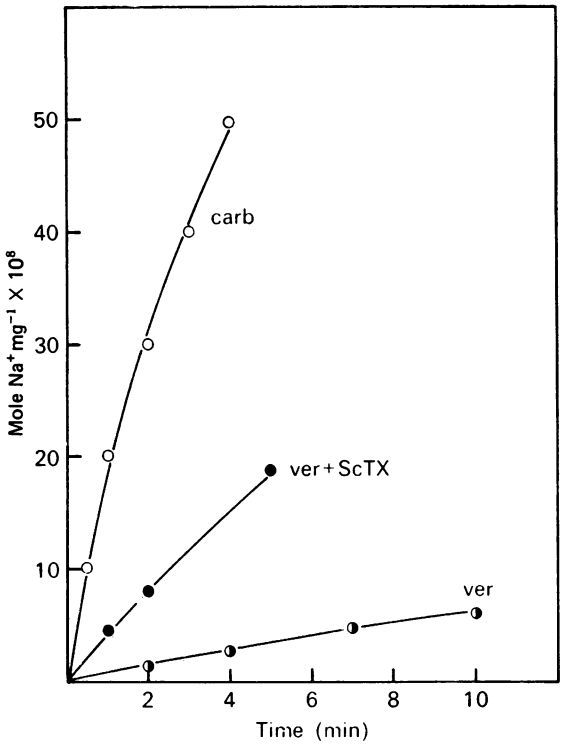

Fig. 1. $^{22}Na^+$ uptake was stimulated by the following reagents. ◑, 2×10^{-4} M-veratridine (ver) at 37 °C. ●, 2×10^{-4} M-veratridine + scorpion venom (ScTX) 100 μg/ml. at 37 °C. ○, 4×10^{-4} M-carbamylcholine (carb) at 22 °C. 5×10^{-3} M-ouabain was present in all assays, and the background uptake caused by ouabain has been subtracted in each case.

(i) *Veratridine and scorpion venom*

Veratridine has been shown in several studies to stimulate $^{22}Na^+$ flux through voltage-dependent Na^+ channels (Catterall & Nirenberg, 1973; Catterall, 1975*b*; Stallcup & Cohn, 1976*a*, *b*). Normally, these channels open only transiently, but veratridine apparently delays the inactivation process (Hille, 1968; Ulbricht, 1969) thus allowing the entry of measurable amounts of $^{22}Na^+$. Scorpion venom also delays Na^+ channel inactivation (Kopenhöfer & Schmidt, 1968; Narahashi, Shapiro, Deguchi, Skuka & Wang, 1972; Romey, Chicheportiche & Lazdunski, 1975). Furthermore, it appears to act cooperatively with veratridine, and in many cases a combination of the two toxins gives a much larger $^{22}Na^+$ flux than either does separately (Catterall, 1975*c*; Stallcup, 1977). Table 1 and Fig. 1 show that 2×10^{-4} M-veratridine stimulates a Na^+ flux of 6×10^{-9} mole min^{-1} mg^{-1} in PC12 and that this is increased to $4{\cdot}2 \times 10^{-8}$ mole min^{-1} mg^{-1} by the addition of scorpion venom. Tetrodotoxin

gives a 50% inhibition of Na^+ uptake at a concentration of 5×10^{-7} M and greater than 98% inhibition at 5×10^{-6} M. These data reflect the presence of voltage-dependent Na^+ channels in PC12, as established by Dichter *et al.* (1977).

Although Catterall (1975*b*) found that divalent cations were competitive inhibitors of veratridine binding in the C1300 mouse neuroblastoma (e.g. 1 mM-Mn^{2+} gave 50% inhibition of the Na^+ flux induced by 10^{-4} M-veratridine), we found that

TABLE 1. $^{22}Na^+$ uptake

Addition	Rate (n-mole min^{-1} mg^{-1})	% inhibition
Veratridine (2×10^{-4} M)	6	—
Veratridine + TTX (5×10^{-6} M)	< 0·1	> 98
Veratridine + Mn^{2+} (2×10^{-3} M)	6	< 1
Veratridine (2×10^{-4} M) + ScTX (100 μg/ml.)	42	—
Veratridine + ScTX + TTX (5×10^{-7} M)	20	52
Veratridine + ScTX + TTX (5×10^{-6} M)	< 1	> 98
Veratridine + ScTX + Mn^{2+} (2×10^{-3} M)	41	2
Carbamylcholine (4×10^{-4} M)	210	—
+ dTC (10^{-4} M)	1	> 99
+ QNB (10^{-4} M)	2	99
+ hexamethonium (10^{-3} M)	10	95
+ αBT (2×10^{-7} M)	200	5
+ anti AChR (1/10 dilution)	10	95
+ TTX (5×10^{-6} M)	210	< 1
+ Mn^{2+} (10^{-3} M)	160	23
(10^{-2} M)	95	55
(4×10^{-2} M)	40	80
+ Co^{2+} (2×10^{-3} M)	172	18
(10^{-2} M)	126	40

Ouabain was present in all assays at a concentration of 5 mM. The rate of $^{22}Na^+$ uptake in the presence of ouabain alone (5 n-mole min^{-1} mg^{-1}) was subtracted from all the rates to obtain the values presented above. dTC, D-tubocurarine; QNB, quinuclidinylbenzilate; αBT, α-bungarotoxin; AChR, acetylcholine receptor; TTX, tetrodotoxin, ScTX, scorpion venom.

Mn^{2+} at a concentration of 2 mM had no effect on $^{22}Na^+$ uptake in PC12 cells. This was true whether Na^+ uptake was induced by veratridine alone or by a combination of veratridine and scorpion venom. As we will see, the differential effects of tetrodotoxin and Mn^{2+} provide the basis for discriminating between voltage-dependent Na^+ and Ca^{2+} channels.

(ii) *Carbamylcholine*

The details of cholinergic Na^+ fluxes in PC12 have been presented elsewhere (Patrick & Stallcup, 1977*a*, *b*), and a summary of this data is presented in Table 1. Briefly, activation of the PC12 acetylcholine receptor cannot be blocked by α-bungarotoxin, but can be blocked by D-tubocurarine, hexamethonium, quinuclidinylbenzilate, and antibody against eel acetylcholine receptor. As shown in Fig. 1 and Table 1, the Na^+ uptake induced by carbamylcholine is extremely rapid: 4×10^{-4} M carbamylcholine (a subsaturating concentration) gives an influx of 2×10^{-7} mole min^{-1} mg^{-1}. To emphasize the fact that this flux occurs through receptor-linked

channels rather than through voltage-dependent ones, data is included in Table 1 which show that 5×10^{-6} M-tetrodotoxin has no effect on the rate of uptake.

The ability of Mn^{2+} and Co^{2+} to block Na^+ flux through the acetylcholine channels can be assessed from the data presented in Table 1. The inhibition produced by Mn^{2+} is examined in more detail in Fig. 4. Relatively large concentrations of Mn^{2+} are required for inhibition: 55% inhibition is achieved at 10 mM-Mn^{2+}, and even at 40 mM-Mn^{2+} inhibition is only 80% complete.

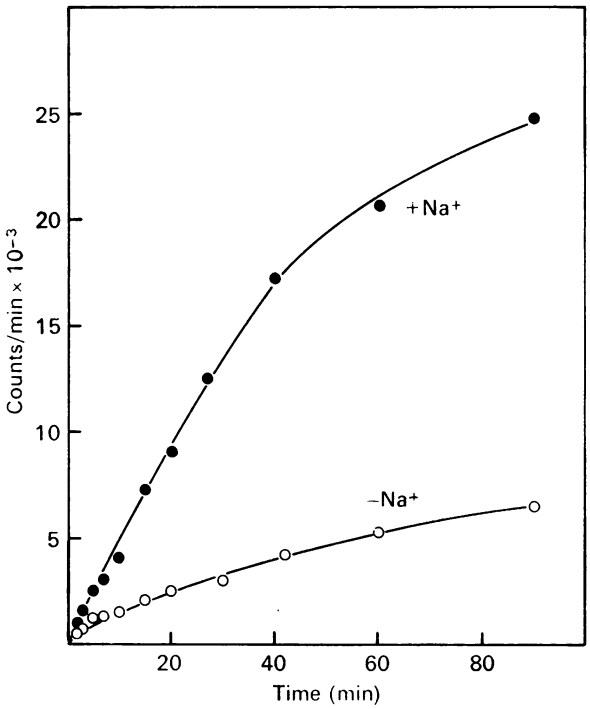

Fig. 2. PC12 cells on polylysine-coated 60 mm dishes were loaded with $^{45}Ca^{2+}$ by treating them for 3 min at 22 °C with 50 mM-K^+ in Na^+-free medium. The plates were quickly washed 3 times with Na^+-free medium and then covered with 4 ml. of either normal saline or Na^+-free medium at 22 °C. 100 μl. aliquots were removed periodically from each dish, centrifuged to remove loose cells, and analysed for $^{45}Ca^{2+}$ content by scintillation counting. ○, $^{45}Ca^{2+}$ efflux in Na^+-free medium. ●, $^{45}Ca^{2+}$ efflux in normal saline.

Calcium uptake

Ouabain was used in our study of $^{45}Ca^{2+}$ fluxes partly for the sake of consistency, i.e. so that the Na^+ and Ca^{2+} fluxes were examined under the same conditions. To make sure that ouabain did not introduce new variables into these Ca^{2+} flux studies we also made several parallel studies in the absence of ouabain. In cases in which $^{45}Ca^{2+}$ uptake was induced by KCl or by carbamylcholine, the omission of ouabain made very little difference in either the rates of uptake or in the patterns of inhibition that we observed. In the case of veratridine and scorpion venom, the omission of ouabain resulted in a decrease in the rate of $^{45}Ca^{2+}$ uptake. However, the properties of this uptake (i.e. its ability to be inhibited by Mn^{2+} or tetrodotoxin) were not

changed. The data reported here were all obtained in the presence of ouabain. Some previous measurements of $^{45}Ca^{2+}$ fluxes in the absence of ouabain were presented elsewhere (Schubert, LaCorbiere, Whitlock, & Stallcup, 1978).

Since there is not an inhibitor of active Ca^{2+} extrusion comparable to ouabain, the possibility that such extrusion competes with uptake during the course of our measurements cannot be ignored. The contribution of a Ca^{2+}-Mg^{2+} pump can probably be discounted since we have omitted Mg^{2+} from our assay medium. Another mechanism for Ca^{2+} extrusion is Na^+-Ca^{2+} exchange. Such a mechanism normally helps to maintain a low intracellular Ca^{2+} level by exchanging intracellular Ca^{2+} for extracellular Na^+ (Blaustein & Oborn, 1975; Rink, 1977). Evidence for the presence of Na^+-Ca^{2+} exchange in PC12 cells was obtained by pre-loading the cells with $^{45}Ca^{2+}$ and measuring the rate of $^{45}Ca^{2+}$ efflux in the presence and absence of extracellular Na^+. Fig. 2 shows that efflux is several times faster in the presence of Na^+ than in its absence, suggesting that extrusion of Ca^{2+} may occur in normal saline (similar experiments in the presence and absence of Ca^{2+} showed no evidence for Ca^{2+}-Ca^{2+} exchange in PC12). Therefore when we try to measure the initial rate of Ca^{2+} uptake in normal saline, we may actually be measuring the sum of influx and efflux. We might also consider whether the Na^+-Ca^{2+} exchange could work in the opposite direction to promote Ca^{2+} uptake; i.e. when cells are loaded with Na^+ during treatment with an effector such as carbamylcholine, could this intracellular Na^+ be exchanged for extracellular Ca^{2+}? Rink (1977) found no evidence that the Na^+-Ca^{2+} exchange in adrenal medulla could be reversed to give Ca^{2+} influx. Furthermore, the available evidence shows that Na^+-Ca^{2+} exchange is insensitive to inhibition by Co^{2+} and Mn^{2+} (Rink, 1977; Benninger, Einwächter, Haas & Kern, 1976). Therefore, even if Ca^{2+} uptake does occur via a reversal of Na^+-Ca^{+2} exchange we should be able to distinguish it from voltage-dependent Ca^{2+} flux which is inhibited by low concentrations of Co^{2+} and Mn^{2+}. We have no direct evidence on whether such a reversal of Na^+-Ca^{2+}. exchange can occur in PC12 cells, but we have tried to rule out the possible reverse exchange not only by doing Mn^{2+} inhibition studies but also by measuring Ca^{2+} uptake both in normal saline and in Na^+-free medium. Clearly, in Na^+-free solution no Na^+ loading can occur, and the rate of $^{45}Ca^{2+}$ uptake by exchange can be no larger in cells stimulated by carbamylcholine, for example, than in the cells tested for background uptake.

(i) K^+

Adrenal chromaffin cells can be maintained in a state of depolarization by raising the extracellular K^+ concentration (Baker & Rink, 1975). Douglas *et al.* (1967*b*) found that 50 mM-K^+ produced a 20 mV depolarization in these cells. Similarly, perfusion of PC12 cells with 30 mM-KCl led to a sustained depolarization of 20 ± 6 mV from a resting potential of -44 ± 5 mV ($\bar{X} \pm$ S.D., $n = 14$ cells; Ritchie, unpublished observations).

A substantial rate of $^{45}Ca^{2+}$ uptake can be induced in PC12 by 50 mM-KCl (Fig. 3*A* and Table 2). This Ca^{2+} flux does not require the presence of Na^+, which is expected if the flux is triggered solely by the K^+-induced depolarization. In fact, the rate of Ca^{2+} uptake is greater in the absence of Na^+ than in its presence. Some possible explanations for this difference are the following. (1) In normal saline the Ca^{2+} extrusion

due to Na^+-Ca^{2+} exchange makes the rate of Ca^{2+} uptake appear smaller than it really is. We have already demonstrated the existence of such an exchange mechanism. (2) In Na^+-free sucrose medium the effective concentration of Ca^{2+} at the cell surface may be increased due to lack of electrostatic screening by Na^+ (Muller & Finkelstein, 1974). This increased availability of Ca^{2+} could enhance the fluxes we measure. (3) The depolarization induced by 50 mM-KCl in Na^+-free medium may be different from that in normal saline. This third alternative has not been tested directly in PC12 cells. We might note, however, that the background rate of $^{45}Ca^{2+}$ uptake (i.e. in 5 mM-KCl) is also enhanced by a factor of 3 in Na^+-free medium (Table 2), a phenomenon that might be explained by alternatives (1) and (2) but not by alternative (3).

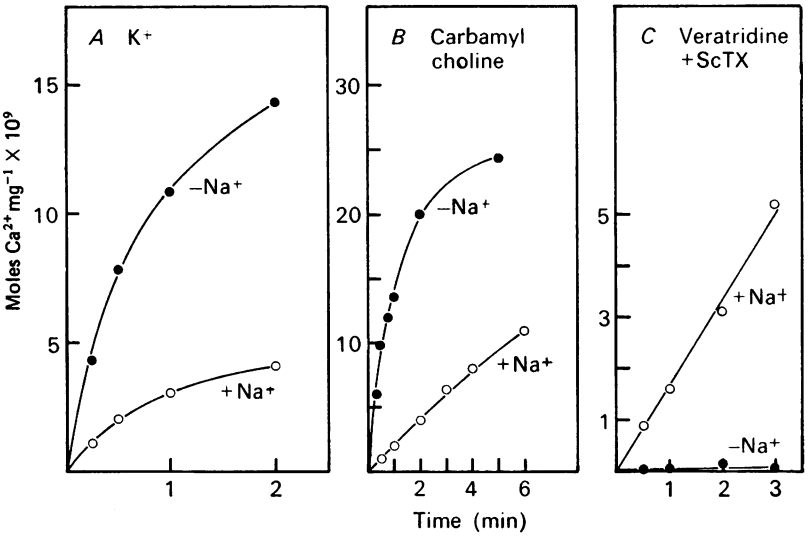

Fig. 3. $^{45}Ca^{2+}$ uptake was induced by the following treatements. *A*, ○, 50 mM-KCl in normal saline at 22 °C. ●, 50 mM-KCl in Na^+-free medium at 22 °C. *B*, ○, 10^{-3} M-carbamylcholine in normal saline at 22 °C. ●, 4×10^{-4} M-carbamylcholine in Na^+-free medium at 22 °C. *C*, ○, 2×10^{-4} M-veratridine + 100 μg/ml. scorpion venom in normal saline at 37 °C. ●, 2×10^{-4} M-veratridine + 100 μg/ml. scorpion venom in Na^+-free medium at 37 °C.

Mn^{2+} and Co^{2+} prove to be very effective inhibitors of K^+-induced Ca^{2+} uptake. Table 2 indicates that 1·0 mM-Mn^{2+} or Co^{2+} gives 95% inhibition of the flux. Such inhibition is characteristic of flux through voltage-dependent Ca^{2+} channels (Hagiwara, 1973). Fig. 4 gives a more detailed picture of the inhibition produced by Mn^{2+}. It is evident that the inhibition is independent of the presence of Na^+. In contrast, 5×10^{-6} M tetrodotoxin, which completely blocks voltage-dependent Na^+ channels, has little or no effect on K^+-induced Ca^{2+} uptake, establishing the separate identities of the voltage-dependent Na^+ and Ca^{2+} channels in PC12.

The mechanism by which Mn^{2+} inhibits voltage-dependent Ca^{2+} uptake seems to be different from that by which tetrodotoxin blocks voltage-dependent Na^+ flux. Tetrodotoxin is thought to block the Na^+ channel without actually passing through it (Hille, 1975). Mn^{2+} appears to compete with Ca^{2+} for passage through the Ca^{2+} channel. Table 3 shows that $^{54}Mn^{2+}$ uptake (in the absence of Ca^{2+}) is stimulated by

treatment of the PC12 cells with 50 mM-KCl, just as is $^{45}Ca^{2+}$ uptake (in the absence of Mn^{2+}). The double label experiment in which both cations are present shows that the rate of uptake of each ion decreases from the rate observed when they were tested separately, suggesting that they compete for entry through the same channel. This is consistent with the observation that Mn^{2+} can support KCl-induced dopamine release in Ca^{2+}-free medium (Ritchie, 1979).

TABLE 2. $^{45}Ca^{2+}$ uptake

Addition	Rate n-mole min^{-1} mg^{-1}	% inhibition
KCl (50 mM) in normal saline	4·9	—
+ Mn^{2+} (10^{-3} M)	0·25	95
+ Co^{2+} (10^{-3} M)	0·23	96
+ TTX (5×10^{-6} M)	4·75	3
KCl (50 mM) in Na^+-free saline	17·0	—
+ Mn^{2+} (10^{-3} M)	0·7	96
+ Co^{2+} (10^{-3} M)	0·6	96
+ TTX (5×10^{-6} M)	16·0	5
Carbamylcholine (10^{-3} M) in normal saline	2·0	—
+ Mn^{2+} (10^{-3} M)	0·24	88
+ TTX (5×10^{-6} M)	2·0	< 1
+ dTC (10^{-4} M)	< 0·05	> 97
Carbamylcholine (4×10^{-4} M) in Na^+-free saline	29·0	—
+ dTC (10^{-4} M)	< 1	> 98
+ αBT (2×10^{-7} M)	27·0	6
+ QNB (5×10^{-5} M)	1·0	97
+ anti AChR (1/10 dilution)	3·0	90
+ TTX (5×10^{-6} M)	28·0	2
+ Mn^{2+} (10^{-3} M)	11·0	62
Veratridine (2×10^{-4} M) + scorpion venom (100 μg/ml.) in normal saline	1·6	—
+ Mn^{2+} (10^{-4} M)	0·8	50
+ Mn^{2+} (2×10^{-4} M)	0·4	75
+ TTX (5×10^{-6} M)	0·07	96
Veratridine (2×10^{-4} M) + ScTX (100 μg/ml.) in Na^+-free saline	< 0·1	—

The background rates of $^{45}Ca^{2+}$ uptake in the presence of ouabain alone were as follows: in normal saline, 0·1 n-mole min^{-1} mg^{-1}; in Na^+-free medium, 0·3 n-mole min^{-1} mg^{-1}. These rates were subtracted in all cases from the total rates to obtain the values presented above.

(ii) *Carbamylcholine*

Acetylcholine (or its analogue carbamylcholine) is known to depolarize adrenal chromaffin cells (Douglas *et al.* 1967*a*, *b*; Brandt *et al.* 1976) and PC12 cells treated with nerve growth factor (Dichter *et al.* 1977). Perfusion of non-NGF-treated cells with 10^{-3} M-carbamylcholine also causes a transient depolarization of 21 ± 6 mV from a resting potential of -44 ± 5 mV ($\bar{X} \pm$ S.D.; $N = 14$ cells; Ritchie, A., unpublished observations). Thus the effect is comparable in magnitude to that produced by 30 mM-KCl (see above).

Carbamylcholine also has the ability to stimulate large $^{45}Ca^{2+}$ fluxes in PC12, as shown in Fig. 3*B* and Table 2. As in the case of high K^+, the Ca^{2+} flux does not

require the presence of Na^+, and once again the rate of uptake is actually larger in the absence of Na^+. However, this time the difference is much larger; more than a tenfold increase is observed in Na^+-free medium. Part of this difference can probably be accounted for by the same factors discussed in the case of high potassium, i.e. the Na^+-Ca^{2+} exchange and the ionic shielding that occur in medium containing Na^+.

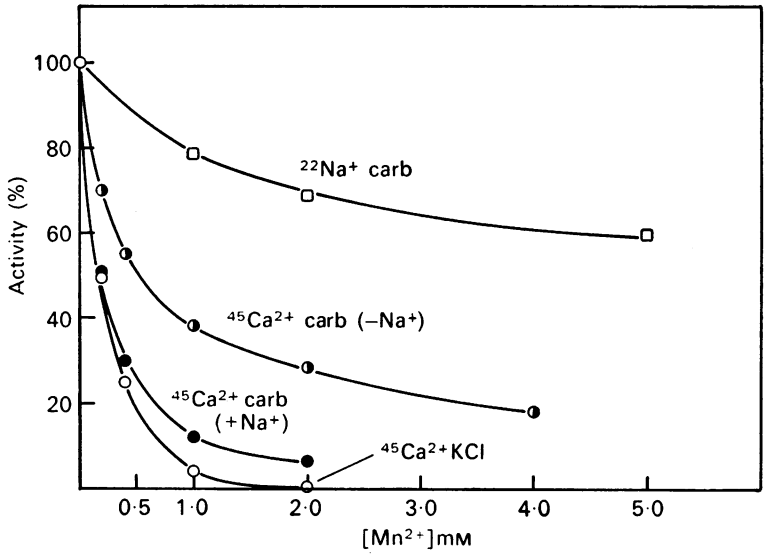

Fig. 4. The indicated concentrations of Mn^{2+} were used to inhibit the following ion fluxes. ○, $^{45}Ca^{2+}$ uptake induced by 50 mM-K^+ in either normal saline or Na^+-free medium at 22 °C. ●, $^{45}Ca^{2+}$ uptake induced by 10^{-3} M-carbamylcholine (carb) in normal saline at 22 °C. ◑, $^{45}Ca^{2+}$ uptake induced by 10^{-3} M-carbamylcholine in Na^+-free medium at 22 °C. □, $^{22}Na^+$ uptake induced by 4×10^{-4} M-carbamylcholine at 22 °C.

TABLE 3

	$^{45}Ca^{2+}$ alone	$^{54}Mn^{2+}$ alone	$^{45}Ca^{2+}$ + $^{54}Mn^{2+}$	
Background rate (n-mole min^{-1} mg^{-1})	0·2	0·29	0·1	0·19
Rate in 50 mM-KCl (n-mole min^{-1} mg^{-1})	5·7	1·23	1·6	0·62
KCl stimulation (n-mole min^{-1} mg^{-1})	5·5	0·94	1·5	0·43
% inhibition in double label	—	—	73%	54%

$^{45}Ca^{2+}$ uptake was determined in Na^+-free medium at 22 °C at a Ca^{2+} concentration of 2 mM. $^{54}Mn^{2+}$ uptake was determined in Na^+-free medium at 22 °C at a Mn^{2+} concentration of 0·5 mM. The double-label experiment was also performed under these same conditions, and the % inhibition values were determined by comparing the rates in the double-label test with the rates obtained when the cations were tested separately.

A clue to the source of the additional increase in Ca^{2+} uptake comes from a study of the Mn^{2+} inhibition data (Fig. 4). In normal saline the Mn^{2+} inhibition profile closely resembles that seen in the case of the KCl-induced Ca^{2+} fluxes. Thus almost all of the Ca^{2+} influx induced by carbamylcholine in normal saline occurs via voltage-dependent Ca^{2+} channels that are triggered by the depolarizing Na^+ influx through the acetylcholine receptor channels. It is important to note, however, that a small

portion of the carbamylcholine-induced Ca^{2+} flux in normal saline is resistant to inhibition by Mn^{2+}. This is most evident at Mn^{2+} concentrations above 1 mM. This resistant component most likely represents a small amount of Ca^{2+} flux through the acetylcholine receptor channels which, as we saw in the case of $^{22}Na^+$ flux, are blocked only at much higher Mn^{2+} concentrations. This receptor-mediated Ca^{2+} flux represents only about 6–8% of the total Ca^{2+} flux observed in normal saline. In Na^+-free sucrose medium the Mn^{2+}-resistant component of Ca^{2+} flux becomes much larger; in 1·0 mM-Mn^{2+} almost 40% of the total carbamylcholine-induced Ca^{2+} flux still occurs. The shape of the inhibition curve at higher Mn^{2+} concentrations closely resembles that seen in the case of $^{22}Na^+$ flux through the receptor channels. Apparently, in the absence of competition from Na^+, Ca^{2+} flux through the receptor channels increases dramatically and probably accounts for the rest of the increase in Ca^{2+} uptake in Na^+-free medium that could not be explained by other means. This receptor-mediated Ca^{2+} flux, even in the absence of any Na^+ flux, may cause sufficient depolarization to trigger the opening of voltage-dependent Ca^{2+} channels. The Mn^{2+} inhibition data suggest that voltage-dependent channels may still account for about 60% of the Ca^{2+} flux in Na^+-free medium. This statement must be qualified somewhat, since attempts to measure depolarization of PC12 cells by perfusion with 10^{-3} M-carbamylcholine in Na^+-free medium containing 10 mM-Ca^{2+} have not been successful (A. Ritchie, unpublished data). A small inward Ca^{2+} current through the acetylcholine channels might, however, escape detection by this method, especially if the cell is damaged significantly by the impalement. In chromaffin cells Douglas *et al.* (1967*b*) were able to measure small (5 mV) depolarizations induced in Na^+-free medium by acetylcholine.

The apparent competition between Na^+ and Ca^{2+} for the acetylcholine channels indicates that there is only one type of ion channel associated with the acetyl-choline receptor, in contrast to the separate voltage-dependent channels for Na^+ and Ca^{2+}. Furthermore, the pharmacology of the carbamylcholine-induced Ca^{2+} uptake in Na^+-free medium is consistent with the idea of a single type of acetylcholine receptor. Table 2 shows that the pattern of inhibition achieved by a α-bungarotoxin, D-tubocurarine, quinuclidinylbenzilate, and antibody to eel acetylcholine receptor is indistinguishable from that observed for carbamylcholine-induced Na^+ uptake in Table 1.

As noted earlier, tetrodotoxin has no effect on carbamylcholine-induced Na^+ flux, and as shown in Table 2 it does not block carbamylcholine-induced Ca^{2+} flux. This indicates that voltage-dependent Na^+ channels do not play a role in the carbamylcholine-induced Ca^{2+} fluxes. Apparently, activation of the voltage-dependent Na^+ channels is not a prerequisite for activation of voltage-dependent Ca^{2+} channels, as suggested by Brandt *et al.* (1976) for chromaffin cells.

(iii) *Veratridine and scorpion venom*

Ulbricht (1969) has shown that veratridine is effective in depolarizing both nerve and muscle. Although we have no data on the effect of veratridine on the membrane potential of PC12 cells, the $^{22}Na^+$ influx stimulated by veratridine and especially by a combination of veratridine and scorpion venom suggests that depolarization probably does occur.

In normal saline the addition of veratridine and scorpion venom causes a Ca^{2+} uptake which is inhibited by low concentrations of Mn^{2+} (Table 2). In order to compare the ability of Mn^{2+} to inhibit veratridine-scorpion venom-induced Ca^{2+} flux with its ability to inhibit KCl-induced flux, the KCl experiments which were previously done at 22 were repeated at 37 °C. Fig. 5 shows that the inhibition of these two processes is virtually identical and that Mn^{2+} appears to inhibit Ca^{2+} flux more effectively at 37 than at 22 °C.

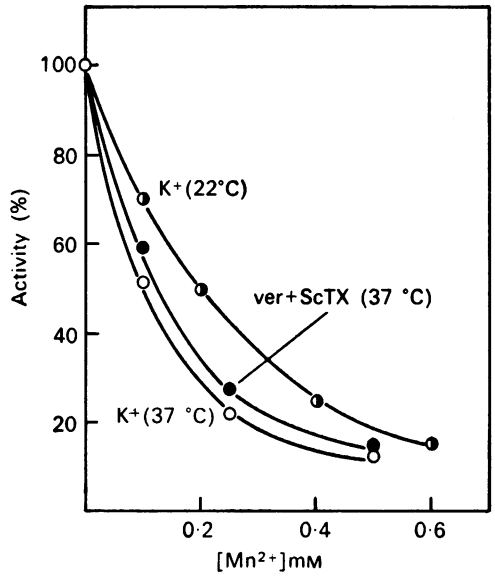

Fig. 5. The indicated concentrations of Mn^{2+} were used to inhibit $^{45}Ca^{2+}$ uptake stimulated in the following ways. ◑, 50 mM-KCl in normal saline at 22 °C. ○, 50 mM-KCl in normal saline at 37 °C. ●, 2×10^{-4} M-veratridine (ver) + scorpion venom (ScTX) 100 μg/ml. in normal saline at 37 °C.

In contrast to K^+ or carbamylcholine-stimulated uptake, the veratridine-scorpion venom-induced Ca^{2+} flux can also be completely blocked by 5×10^{-6} M-tetrodotoxin (Table 2), which is specific for voltage-dependent Na^+channels. These data indicate that in the case of veratridine and scorpion venom the primary event is a Na^+ flux through voltage-dependent Na^+ channels. This flux apparently depolarizes the cell sufficiently to trigger a secondary Ca^{2+} flux through voltage-dependent Ca^{2+} channels which can be blocked by Mn^{2+}. This scheme is borne out by the finding that in Na^+-free medium veratridine and scorpion venom do not stimulate a detectable Ca^{2+} flux. These data establish two points: (1) Ca^{2+} does not pass through the voltage-dependent Na^+ channels and (2) veratridine and scorpion venom do not activate the voltage-dependent Ca^{2+} channels.

DISCUSSION

We have presented evidence for three types of ion channels in PC12 cells: (1) voltage-dependent Na^+ channels, (2) voltage-dependent Ca^{2+} channels, and (3) acetylcholine receptor-linked channels that are permeable to both Na^+ and Ca^{2+}.

Voltage-dependent Na^+ channels

Several previous studies with cloned nerve and muscle cell lines have shown that veratridine and scorpion venom can be used to study voltage-dependent or action-potential Na^+ channels (Catterall & Nirenberg, 1973; Catterall, 1975*b*; Catterall, 1975*c*; Stallcup & Cohn, 1976*a*, *b*; Stallcup, 1977). In the case of PC12 cells the rate of $^{22}Na^+$ uptake induced by veratridine could be increased by a factor of 7 by the addition of scorpion venom. The contention that these Na^+ fluxes occur via voltage-dependent Na^+ channels is supported by the finding that tetrodotoxin is able to block the fluxes. 5×10^{-7} M-tetrodotoxin decreases the rate of $^{22}Na^+$ uptake by 50%, while 5×10^{-6} M-TTX completely blocks uptake. These concentrations are 20 to 50-fold higher than the concentrations of tetrodotoxin needed to block Na^+ flux in other nerve cell lines we have examined (Stallcup, 1977). The relative insensitivity of PC12 to tetrodotoxin more closely matches that of cultured muscle cells. Although divalent cations at concentrations of 1–5 mM have been reported to compete effectively for the veratridine binding sites in mouse neuroblastoma (Catterall, 1975*b*) and in cardiac muscle (Fosset, deBarry, Lenoir & Lazdunski, 1977), in our hands Mn^{2+} up to a concentration of 2 mM has no effect on the Na^+ uptake induced in PC12 by veratridine or by veratridine and scorpion venom.

Voltage-dependent Ca^{2+} channels

PC12 cells, as well as chromaffin cells, have been shown to release catecholamines in response to several types of depolarizing stimuli, suggesting the presence of voltage-dependent Ca^{2+} channels. Elevated levels of extracellular K^+ (Vogt, 1952; Douglas & Rubin, 1963; Baker & Rink, 1975; Chalfie *et al.* 1976; Greene & Rein, 1977*a*, *b*), acetylcholine or carbamylcholine (Douglas *et al.* 1967*a*, *b*; Greene & Rein, 1977*c*; Ritchie, 1979), and veratridine (Greene & Rein, 1977*a*) have been shown to be effective in stimulating catecholamine secretion. We find that each of these conditions induces $^{45}Ca^{2+}$ uptake that can be blocked by low concentrations of Mn^{2+} and Co^{2+}, indicative of the involvement of voltage-dependent Ca^{2+} channels (Hagiwara, 1973). It is interesting to note here that catecholamine secretion is not the only physiological function stimulated by these Ca^{2+} fluxes. An increase in intracellular Ca^{2+} induced by any of the above conditions causes improved cell-substratum adhesion and neurite extension in PC12 (Schubert *et al.* 1978). Nerve growth factor (NGF), which causes neurite outgrowth in PC12 (Greene & Tischler, 1976), also causes a mobilization of intracellular Ca^{2+}.

The very fact that we can measure substantial $^{45}Ca^{2+}$ flux through voltage-dependent Ca^{2+} channels under a variety of conditions provides a clue to another property of the PC12 Ca^{2+} channels. Namely, they must inactivate very slowly or not at all. By comparison, $^{22}Na^+$ flux through voltage-dependent Na^+ channels, which inactivate very quickly, can be measured only in the presence of veratridine. In the absence of veratridine the Na^+ channels open so transiently that neither in high K^+ (Stallcup, unpublished observation) nor in carbamylcholine do we see a tetrodotoxin sensitive component of $^{22}Na^+$ uptake. The details of the PC12 Ca^{2+} channel inactivation or failure to inactivate remain to be resolved.

Voltage-dependent $^{45}Ca^{2+}$ fluxes have also been observed in other systems. In rat

brain synaptosomes, veratridine and scorpion venom have been shown to stimulate $^{45}Ca^{2+}$ uptake and to cause release of noradrenalin (Blaustein, 1975). These effects could be blocked by tetrodotoixn or by replacement of Na^+ with choline. High K^+ also stimulated both Ca^{2+} uptake and transmitter release, and as expected, these effects were sensitive to Mn^{2+}, insensitive to tetrodotoxin, and not abolished by removal of Na^+. Previously, we measured depolarization-induced $^{45}Ca^{2+}$ uptake in the clonal skeletal muscle cell line L6 (Stallcup & Cohn, 1976*a*). As in PC12, the necessary depolarization could be produced by 50 mM-KCl, by veratridine in medium containing Na^+, or by carbamylcholine, and in each case $^{45}Ca^{2+}$ uptake was blocked by low Mn^{2+} concentrations. Tetrodotoxin was able to block $^{45}Ca^{2+}$ uptake only in the case of veratridine treatment, which as we have seen in the PC12 cells, is dependent on a primary flow of Na^+ ions through the voltage-dependent Na^+ channels. Fosset *et al.* (1977) have reported that veratridine causes both $^{22}Na^+$ and $^{45}Ca^{2+}$ uptake in cultured embryonic heart cells. Once again this Ca^{2+} uptake was dependent on the presence of Na^+ and could be blocked by tetrodotoxin. However, Mn^{2+} and Co^{2+} were unable to inhibit the Ca^{2+} flux in these cells. Thus Ca^{2+} uptake by the cardiac cells may occur via Na^+-Ca^{2+} exchange which, as we stated earlier, is insensitive to inhibition by Mn^{2+} and Co^{2+}. Indeed such a mechanism has been postulated to be of great importance in heart muscle (Benninger *et al.* 1976). These findings indicate an important difference between the mode of Ca^{2+} entry into cardiac cells and into PC12 cells and reinforce our contention that in PC12 we are dealing with a voltage-dependent Ca^{2+} channel.

Acetylcholine receptor channels

The detailed pharmacology of the PC12 acetylcholine receptor was previously investigated by studying the $^{22}Na^+$ flux resulting from activation of the receptor (Patrick & Stallcup, 1977*a*, *b*). Activation of this receptor could be blocked by a variety of nicotinic cholinergic antagonists, with the exception of α-bungarotoxin. Thus this receptor appears to differ from other acetylcholine receptors that have been studied by means of $^{22}Na^+$ flux measurements. Carbamylcholine-induced $^{22}Na^+$ uptake in primary cultures of chick muscle (Catterall, 1975*a*), in the clonal skeletal muscle cell line L6 (W. B. Stallcup, unpublished observation), and in the non-fusing clonal cell line BC_3H-1 (Patrick & Stallcup, 1977*a*) can be virtually abolished by 10^{-8} M-α-bungarotoxin.

Our data indicate that, while in normal medium only a small amount of Ca^{2+} enters the cell via the acetylcholine channels, in Na^+-free (sucrose substituted) medium a substantial amount of Ca^{2+} enters through these channels. This Ca^{2+} flux can be blocked by D-tubocurarine, hexamethonium, quinuclidinylbenzilate, and antibody to eel acetylcholine receptor, but not by α-bungarotoxin. This is the same pharmacology observed for cholinergic $^{22}Na^+$ uptake in PC12. Thus these data are consistent with the idea that there is only one type of acetylcholine receptor on PC12 and that it can mediate permeability to both Na^+ and Ca^{2+}.

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