Biochimica et Biophysica Acta, 648 (1981) 55–62
Elsevier/North-Holland Biomedical Press

BBA 79399

EFFECTS OF 2-CHLOROADENOSINE ON ELECTRICAL POTENTIALS IN BRAIN SYNAPTIC MEMBRANE VESICLES

MARY L. MICHAELIS and ELIAS K. MICHAELIS

Neurobiology Section, Department of Human Development and Drug Design Program University of Kansas, Lawrence, KS 66045 (U.S.A.)

(Received April 10th, 1981)

Key words: Neuronal membrane; Lipophilic cation; Adenosine analog; K^+ flux; Membrane potentials; (Rat brain)

Isolated synaptic plasma membrane vesicles developed an internal negative membrane potential ($\Delta\psi$) following loading with potassium succinate and incubation in NaCl, sodium succinate, or Tris succinate media. Membrane $\Delta\psi$ was monitored by measuring triphenyl[^{3}H]methylphosphonium ion ([^{3}H]TPMP$^+$) accumulation by these vesicles. Estimates of $\Delta\psi$ ranged from −6.9 mV for vesicles incubated in sodium succinate to −28 mV for membranes incubated in NaCl. Intravesicular TPMP$^+$ accumulation was strongly dependent on the K^+ diffusion potential and was enhanced by the K^+ ionophore valinomycin and by the adenosine analog 2-chloroadenosine (2-Cl-Ado). The stimulation of TPMP$^+$ influx by 2-Cl-Ado was dependent on the concentration of this agent, independent of Cl^- fluxes, and sensitive to inhibition by the methylxanthine theophylline. The increase in $\Delta\psi$ of the synaptic membrane vesicles caused by 2-Cl-Ado paralleled the hyperpolarization of neurons produced by adenosine and 2-Cl-Ado in physiological systems.

Adenosine and a number of adenosine analogs and adenine nucleotides have a depressant action on nerve cell excitability and neuronal transmission in the central and peripheral nervous systems (e.g., Refs. 1–6). The neuroinhibitory activity of adenosine appears to be due primarily to inhibition of the release of excitatory transmitters rather than to direct inhibition of postsynaptic neurons and muscle cells [1,2]. Inhibition of transmitter release by adenosine has been demonstrated for both adrenergic and cholinergic innervation in the peripheral nervous system [6–8]. A similar action by adenosine and its less-readily metabolized analog 2-chloroadenosine (2-Cl-Ado) [9] has also been shown with respect to depolarization-induced dopamine, acetylcholine, and 5-hydroxytryptamine release from brain slices [10] and dopamine release from isolated brain nerve ending particles (synaptosomes) [11]. It remains unclear at this point whether adenosine inhibits depolarization-induced transmitter release from presynaptic nerve endings by altering the presynaptic membrane potential ($\Delta\psi$) or by directly affecting the influx of Ca^{2+} into the nerve terminals [12]. In the present study, we have attempted to measure the effects of 2-Cl-Ado on the synaptic membrane potential.

Detection of shifts in $\Delta\psi$ in synaptosomal preparations has been accomplished through the use of voltage-sensitive carbocyanine dyes [13] and through measurement of the passive distribution of the lipophilic cation tetraphenylphosphonium (TPP$^+$) [14]. Both techniques have led to estimates of $\Delta\psi$ in synaptosomes in the range of −55 to −80 mV [13, 14]. Estimates of $\Delta\psi$ obtained through direct measurement of the distribution of Na^+, K^+, and Cl^- in synaptosomes were somewhat lower than those detected with the other techniques, −26 to −30 mV [15].

Recent studies from several laboratories have demonstrated that many of the essential properties of

Abbreviations: TPMP$^+$, triphenylmethylphosphonium ion; TPP$^+$, tetraphenylphosphonium ion; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; 2-Cl-Ado, 2-chloroadenosine.

brain synaptosomes such as Na^+-dependent transmitter uptake [16], transmitter-induced Na^+ flux [17], and Na^+-Ca^{2+} antiporter activity [18] are as active in isolated released synaptic plasma membrane vesicles as they are in intact synaptosomes. In this report, we have used the lipophilic cation triphenyl-[^{3}H]methylphosphonium ([^{3}H]TPMP$^+$) in order to determine whether these purified resealed synaptic plasma membrane vesicles have the capacity to develop and maintain a membrane potential and whether 2-Cl-Ado affects this transmembrane potential.

Methods and Materials

Preparation of synaptic membrane vesicles. The resealed synaptic membrane vesicles from brain were prepared as described previously [17,18]. The synaptic membranes were suspended in 0.25 M sucrose/14 mM potassium succinate/50 μM $MgCl_2$, pH 7.4, at a final protein concentration of 9–12 mg/ml, divided into small aliquots, quickly frozen in liquid N_2, and stored at −80°C for periods no longer than 3 weeks. Protein concentrations of membrane preparations were determined according to Lowry et al. [19]. The purity of the membrane vesicle preparation was monitored through determinations of the activity of the plasma membrane enzyme ($Na^+ + K^+$)-ATPase as described previously [17] and by electron microscopy. Membrane samples used for electron microscopy were fixed overnight at 4°C in 5% glutaraldehyde, 0.04% $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.0. The samples were stained with 4% OsO_4, sectioned, and stained with uranyl acetate.

TPMP$^+$ uptake assays. Aliquots of frozen vesicles were thawed rapidly at 37°C for 10 min in the presence of 6 vol. 75 mM potassium succinate/50 μM $MgCl_2$, pH 7.4. The potassium succinate-loaded vesicles were incubated for an additional 10 min at 23°C prior to the initiation of the uptake assay. When the effects of 2-Cl-Ado were being studied, varying concentrations of this agent were added during this preincubation period. [*Me*-^{3}H]TPMP([^{3}H]TPMP$^+$) uptake was measured by incubating the membrane vesicles (60–80 μg protein) in 300 μl final volume of a reaction mixture containing either 110 mM NaCl or KCl, 0.5 mM [^{3}H]TPMP$^+$ (0.25 μCi), 20 mM Tris-HCl, pH 7.4. As indicated for some experiments, 75 mM sodium succinate, potassium succinate, or Tris succinate were used in place of the NaCl and KCl. All incubations were carried out at 23°C for the specified time periods. Incubations were terminated by dilution of the samples with 2 ml of cold 0.4 M NaCl or 0.4 M choline chloride in 10 mM Tris-HCl, pH 7.4, and immediate filtration through Millipore filters (HAWP 0.45 μ). The filters were washed with 3 ml of the 'stop' solution used to terminate the incubations. Dilution, filtration and washing were usually complete within 5 s. Nonspecific adsorption of [^{3}H]-TPMP$^+$ to the filters was determined in samples to which 2 ml of cold 'stop' solution were added prior to the addition of the membranes. These samples were then filtered and washed identically to the others, and the values obtained in this way were subtracted from the values for the incubated samples in the calculation of TPMP$^+$ uptake. Estimates of the intravesicular volume of synaptosomes and synaptic plasma membrane vesicles have yielded an average value of approx. 3.6 μl/mg protein [14,15,17]. This figure was used in calculations of $\Delta\psi$ using the Nernst equation as follows:

$$\Delta\psi = RT/nF \ln \frac{[\text{TPMP}]_{in}}{[\text{TPMP}]_{out}}$$

Materials. Triphenyl[^{3}H]methylphosphonium bromide (3.59 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA. The valinomycin, gramicidin D, 2-Cl-Ado, and theophylline were purchased from Sigma Chemical Co., St. Louis, MO, the triphenylmethylphosphonium bromide from ICN Pharmaceuticals, Plainview, NY and the 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) from Calbiochem-Behring Corp., LaJolla, CA. Deoxycoformycin was generously provided by Dr. Henry Dion, Warner-Lambert, Detroit, MI and the dipyridamole by Boehringer-Ingleheim, Ltd., Ridgefield, CT.

Results and Discussion

[^{3}H]TPMP$^+$ accumulation by synaptic membrane vesicles

The structural characteristics of the synaptic plasma membranes employed in the study of [^{3}H]TPMP$^+$ accumulation were determined by electron microscopy (Fig. 1). Examination of three membrane batches

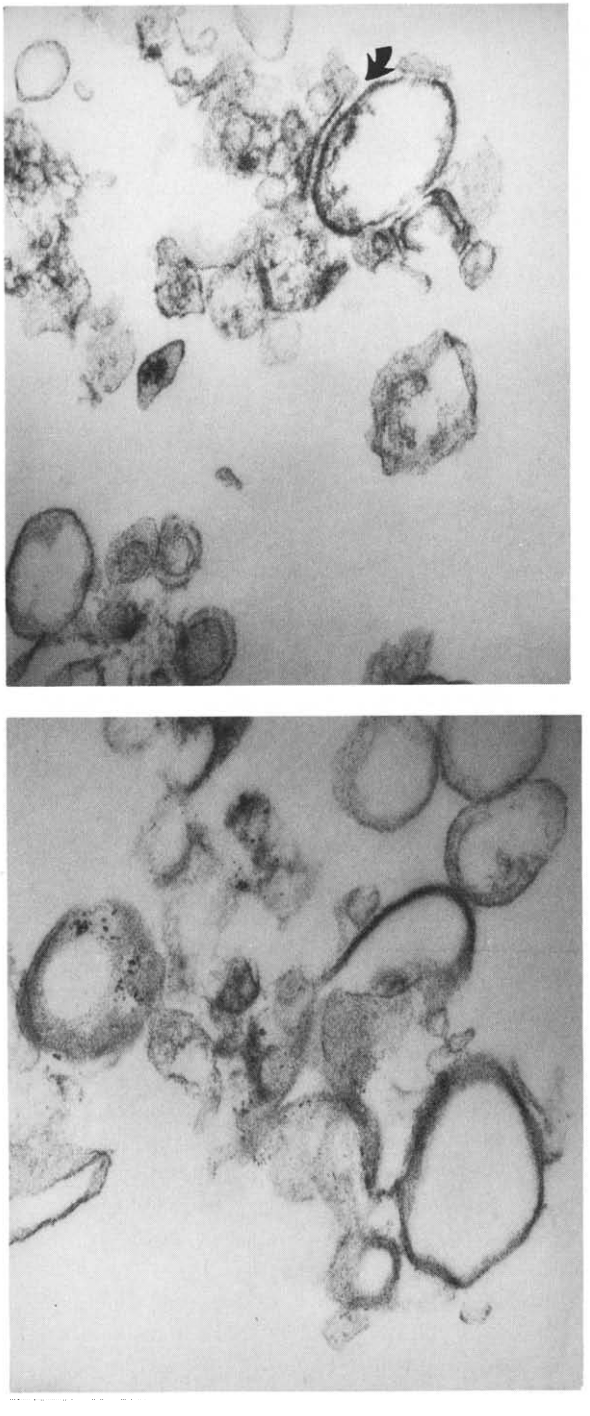

Fig. 1. Electron photomicrographs of one of the resealed synaptic plasma membrane preparations. The arrow in the top panel indicates a representative synaptic junctional complex. Magnification 55 000X.

and several electron microscopic fields revealed that these preparations were enriched in empty membrane sacs (approx. 70–75% of all structures). In addition, pre- and postsynaptic membrane junctions were easily identified in almost every field (Fig. 1). These membrane preparations also exhibited a greater than 2-fold increase in the specific activity of the plasma membrane marker enzyme ($Na^+ + K^+$)-ATPase as compared to the enzyme activity in intact synaptosomes [17,18].

Synaptic membrane vesicle resealed in the presence of 75 mM potassium succinate and incubated in either a NaCl or a KCl medium accumulated [^{3}H]-$TPMP^+$ quite rapidly (Fig. 2). The accumulation of $TPMP^+$ by the membrane vesicles in the presence of a KCl incubation medium was consistently found to be slower and the magnitude generally less than half that taken up by vesicles incubated in a NaCl medium (Fig. 2). The uptake of $TPMP^+$ under the KCl incubation conditions was thought to represent either passive chemical equilibration of the cation into the intravesicular space or possibly $TPMP^+$ uptake as a result of the Cl^- diffusion potential which was present under these conditions. With an estimated synaptic membrane vesicular volume of 3.6 μl/mg protein

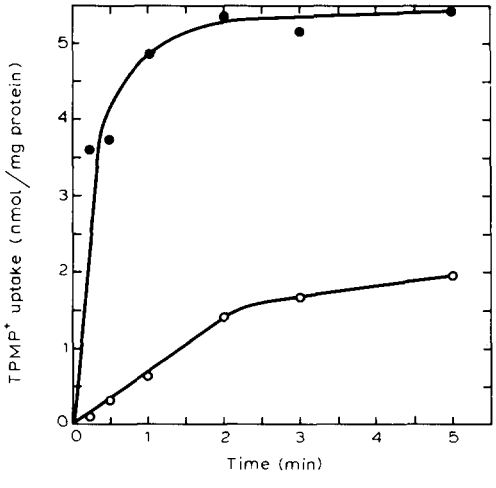

Fig. 2. Time course of $TPMP^+$ uptake into synaptic membrane vesicles. The potassium succinate-loaded vesicles were incubated in either 110 mM NaCl (•) or 110 mM KCl (○), and all incubations were terminated with 0.4 M NaCl/10 mM Tris-HCl. Each point represents the mean of 4–8 determinations from two preparations and the S.E. of the means were less than 15% of the mean values.

[14], the intravesicular concentration of $TPMP^+$ at equilibrium in the presence of KCl would be 0.55 mM (5 min in Fig. 2), which is only slightly higher than the extravesicular concentration of $TPMP^+$ (0.5 mM). On the other hand, the intravesicular $TPMP^+$ concentration at equilibrium (1 min) in vesicles incubated in the presence of 110 mM NaCl was 1.53 mM, or approx. 3-times the extravesicular concentration. Assuming that $TPMP^+$ was distributed passively according to the $\Delta\psi$ across the synaptic plasma membranes, then the $\Delta\psi$ can be calculated to be equal to −28.2 mV under the NaCl incubation condition and −2.4 mV under the KCl condition. The difference between the values of $\Delta\psi$ in the absence of K^+ in the external medium is indicative of the dependence of the synaptic membrane $\Delta\psi$ on the K^+ diffusion potential, as has previously been described for synaptosomes [14]. A notable difference between the determination of $\Delta\psi$ in synaptosomes and in isolated synaptic membrane vesicles by means of lipophilic cation distribution was the observation that the synaptosomes had a relatively large residual TPP^+ accumulation, even in the presence of a high external K^+ concentration (193 mM) [14]. It should be pointed out, however, that the $\Delta\psi$ determined in the resealed synaptic membrane vesicles is only a reflection of the artificially imposed ionic gradients and does not represent the actual $\Delta\psi$ of intact nerve endings.

Effects of ionophores on $TPMP^+$ accumulation in synaptic membrane vesicles

The uptake of $TPMP^+$ in the presence of NaCl was markedly enhanced by addition of the K^+ ionophore valinomycin (Fig. 3). For the vesicles incubated in NaCl, valinomycin would be expected to induce an increase in the K^+ flux from intra- to extravesicular space, and this should cause the interior of the vesicles to become more negative due to the lesser permeability of the anions (succinate) remaining behind. As can be seen in Fig. 3, 0.1 μM valinomycin rapidly brought about a near doubling of the $TPMP^+$ uptake into vesicles incubated in the NaCl medium, while it had virtually no effect on vesicles incubated in a KCl medium. The $\Delta\psi$ for this particular preparation at steady state in the absence of valinomycin was −16.4 mV, whereas in the presence of valinomycin it reached a value of −30.3 mV. The steady state level

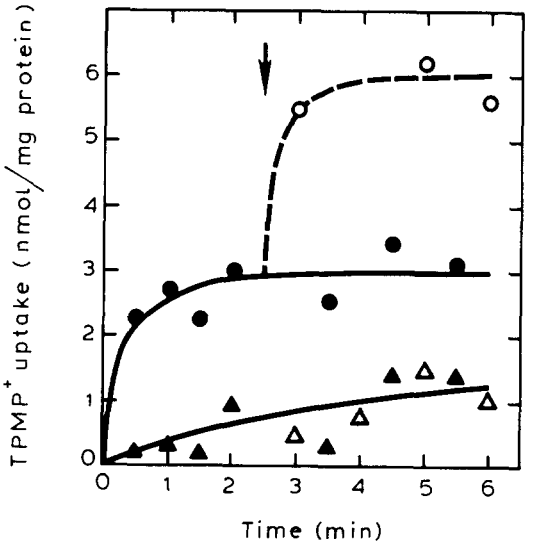

Fig. 3. The effect of the K^+ ionophore valinomycin on $TPMP^+$ uptake. The potassium succinate-loaded vesicles were incubated in 110 mM NaCl with (○) or without (●) the addition of 0.1 μM valinomycin, or in 110 mM KCl with (△) or without (▲) 0.1 μM valinomycin. Incubations were stopped with 0.4 M NaCl/10 mM Tris-HCl. At the time indicated by the arrow, valinomycin in 10% methanol was added to the experimental samples and a comparable volume of 10% methanol was added to the controls. The points represent the means of duplicate determinations with a single membrane preparation.

of intravesicular $TPMP^+$ achieved in the presence of valinomycin should no longer be dependent upon K^+ diffusion gradients. Thus, the enhanced $TPMP^+$ accumulation at steady state suggests that the membranes were relatively impermeable to the other ionic species in the medium, at least for the brief period of time during which $TPMP^+$ distribution was monitored. The results obtained under these conditions indicate that addition of valinomycin did not lead to an immediate influx of Na^+, unless that Na^+ influx was electroneutral, i.e., balanced by an equivalent influx of a negatively charged species such as Cl^-.

A decrease in intravesicular accumulation of [^{3}H]-$TPMP^+$ was observed when the monovalent cation ionophore gramicidin D was added after 1 min of incubation (Fig. 4). The $TPMP^+$ uptake assay conditions in this experiment differed from the previous incubation procedures. A 0.4 M choline chloride 'stop' and 'wash' solution was used in place of the 0.4 M NaCl medium in order to avoid the influx of

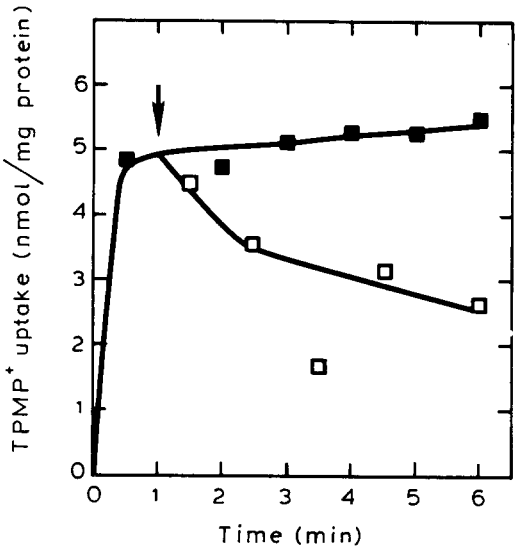

Fig. 4. The effect of the Na^+ ionophore gramicidin D on $TPMP^+$ accumulation into synaptic membrane vesicles. Potassium succinate-loaded vesicles were incubated in 56 mM NaCl/72 mM KCl in the presence (□) or the absence (■) of 0.1 μM gramicidin D. The arrow indicates the point at which gramicidin D in methanol was added to the experimental samples and a comparable volume of methanol was added to the controls. All incubations were stopped with 0.4 M choline chloride/10 mM Tris-HCl. Each point is the mean of triplicate determinations from a single membrane preparation in which the S.E. were less than 10% of the mean values.

Na^+ from this solution, especially in the presence of gramicidin D. The use of choline chloride in place of the NaCl 'stop' solution was found to enhance $TPMP^+$ accumulation. A second change introduced in these assays involved the incubation of the vesicles in a medium of lower Na^+ and K^+ gradients as described in the legend for Fig. 4. The addition of 0.1 μM gramicidin D under these conditions led to a progressive loss of $TPMP^+$ from the intravesicular space. The $\Delta\psi$ at 6 min of incubation was −28.1 mV in the absence of gramicidin D and −9.5 mV in the presence of the ionophore. Since gramicidin D would permit both Na^+ and K^+ to traverse the membrane relatively easily, it is assumed that as K^+ moved down its chemical gradient from the intravesicular to the extravesicular space, gramicidin D facilitated the inward flux of Na^+ down its electrochemical gradient. This Na^+ influx then balanced the internal negativity created by the K^+ efflux and the transmembrane potential was gradually reduced. Unlike the situation with the K^+-selective ionophore valinomycin, the presence of gramicidin also enhances Na^+ permeability, and it appears that the electrochemical gradients for Na^+ influx did lead to dissipation of the membrane potential following the introduction of this ionophore. The results of these experiments also indicated that $TPMP^+$ was fully mobile and that its distribution within this system was determined by the existing electrochemical gradient across the membranes.

Effects of 2-Cl-Ado on [^{3}H]TPMP$^+$ accumulation

The adenosine analog 2-Cl-Ado is known to be at least as potent as adenosine in stimulating the activity of neuronal adenylate cyclases [21,22], in causing inhibition of neuronal excitation [1,2], and in inhibiting the release of neurotransmitters from presynaptic nerve terminals [11]. Therefore, 2-Cl-Ado was used in most of the explorations of the effects of purine nucleosides on synaptic membrane $\Delta\psi$ as determined by $TPMP^+$ distribution. Since it was possible that the purine-induced hyperpolarization of neuronal membranes could be due to an enhancement of either K^+ or Cl^- diffusion potentials [12], the initial studies with 2-Cl-Ado were conducted in the absence of Cl^- in the incubation media.

As can be seen in Fig. 5A and B, preincubation of the synaptic membrane vesicles with 10 μM or with 25 μM 2-Cl-Ado led to a substantial increase in the initial rate of [^{3}H]$TPMP^+$ uptake into potassium succinate-loaded vesicles. The stimulation of the inital $TPMP^+$ uptake into potassium succinate-loaded vesicles was observed in membranes which were incubated in a sodium succinate (Fig. 5A) and in a Tris-succinate medium (Fig. 5B). It can also be observed in Fig. 5A and B that 2-Cl-Ado had no effect on $TPMP^+$ accumulation into vesicles which were incubated in a potassium succinate medium, i.e., in the absence of K^+ diffusion potentials.

When potassium succinate-loaded membranes were incubated in the Tris succinate medium, the only readily permeant ions through the plsama membranes were the potassium ions. The increase in $TPMP^+$ accumulation observed following exposure of these membranes to 2-Cl-Ado (Fig. 5B), was most likely due to a stimulation of the K^+ diffusion potential. The fact that 2-Cl-Ado had no effect on $TPMP^+$ accumulation when the membranes were incubated in a

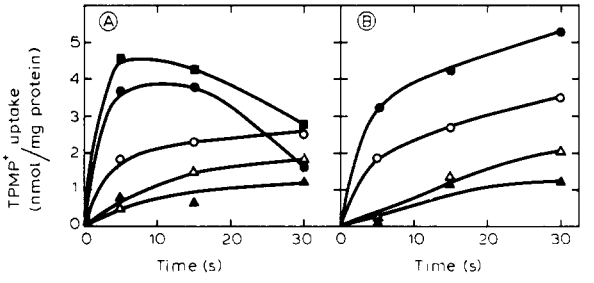

Fig. 5. The effect of 2-Cl-Ado on $TPMP^+$ accumulation by synaptic membrane vesicles. The vesicles were loaded internally with potassium succinate and incubated in various [^{3}H]$TPMP^+$-containing media for the time periods indicated. (A) Membrane vesicles were incubated in 75 mM sodium succinate (○), in 75 mM sodium succinate after preincubation with 10 μM 2-Cl-Ado (■) or with 25 μM 2-Cl-Ado (●), in 75 mM potassium succinate (△), or in 75 mM potassium succinate following preincubation with 25 μM 2-Cl-Ado (▲). All incubations were stopped with 0.4 M sodium succinate, pH 7.5. Each point is the mean of 6–10 determinations from three membrane preparations with S.E. less than 15% of the mean values. (B) Membrane vesicles were incubated in 75 mM Tris succinate (○), in 75 mM Tris succinate following preincubation with 25 μM 2-Cl-Ado (●), in 75 mM potassium succinate (△), or in 75 mM potassium succinate following preincubation with 25 μM 2-Cl-Ado (▲). All incubations were stopped with 0.4 M Tris succinate, pH 7.5. Each point is the mean of 6–8 deteminations from two membrane preparations with S.E. less than 18% of the mean values.

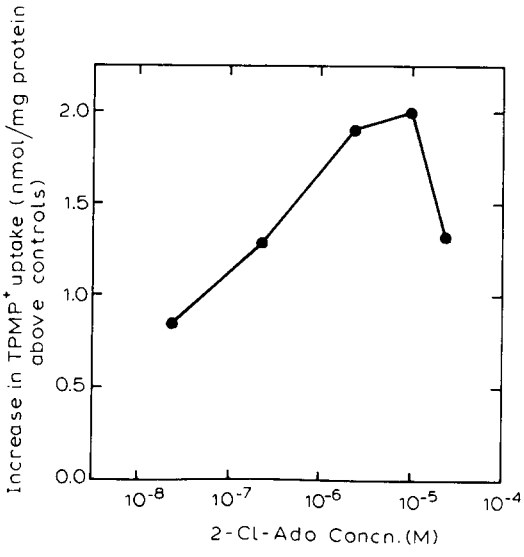

Fig. 6. Dose-response characteristics of the effects of 2-Cl-Ado on $TPMP^+$ uptake. The potassium succinate-loaded vesicles were preincubated with the indicated concentrations of 2-Cl-Ado. The vesicles were then incubated with [^{3}H]$TPMP^+$ in 75 mM sodium succinate for 5 s and incubations were terminated with 0.4 M sodium succinate. The data are plotted as the increase in $TPMP^+$ uptake above that in control samples. The average uptake in the control samples was 2.047 ± 0.20 (n = 10). Each point represents the mean of 5–6 determinations with S.E. which were less than 15% of the mean value.

potassium succinate medium (Fig. 5A and B) was also suggestive of the dependence of the 2-Cl-Ado-induced increase in $\Delta\psi$ on an enhancement of the K^+ diffusion potential. Furthermore, the latter observation would indicate that 2-Cl-Ado did not increase $TPMP^+$ association with the synaptic membrane vesicles through an increase in $TPMP^+$ binding, but apparently through an effect on the membrane $\Delta\psi$.

The differential levels of $TPMP^+$ accumulation caused by 2-Cl-Ado were usually dissipated after about 30–60 s of incubation in sodium succinate medium, whereas they remained rather stable even after 60 s of incubation in Tris succinate medium (Fig. 5A vs. B). The greater stability of the effect of 2-Cl-Ado on $TPMP^+$ accumulation when measured in a Tris succinate medium with a Tris succinate stop solution may have been due to the lesser permeability of Tris ions through the synaptic membranes as compared to the permeability of Na^+. As was shown with gramicidin D, Na^+ influx into the intravesicular space would be expected to bring about a progressive loss of the intravesicular $TPMP^+$.

Exposure of the synaptic membranes to 25 μM 2-Cl-Ado led to the development of $\Delta\psi$ (internal negative) of −17.8 and −16.02 mV at 5 s of incubation in sodium succinate and Tris succinate media, respectively (Fig. 5A and B). The $\Delta\psi$ of control membranes in the same incubation media at 5 s was −6.9 mV. Preincubation with 10 μM 2-Cl-Ado caused an even greater change in $\Delta\psi$ (−23.6 mV) than 25 μM 2-Cl-Ado produced in vesicles incubated in sodium succinate for 5 s. This concentration of 2-Cl-Ado appeared to be the optimal one for stimulation of $TPMP^+$ uptake in membrane vesicles incubated under the sodium succinate conditions as is shown in Fig. 6. The concentration of 2-Cl-Ado which brought about half-maximal activation of $TPMP^+$ influx was estimated from log probit analysis of the data in Fig. 6, and was found to be equal to 54.9 nM. This value was quite close to the concentration of 2-Cl-

Ado which produced 50% inhibition of dopamine release from stratial synaptosomes (15 nM) [11], and within the range of the estimated K_D for 2-[^{3}H]chloroadenosine binding to brain synaptic membranes (16–23.5 nM) [23,24].

Preincubation of the synaptic membrane vesicles with the natural nucleoside adenosine (25 μM) also led to an increase in $\Delta\psi$ for vesicles incubated for 5 s in sodium succinate. The value of −12.8 mV for the control membranes was increased to −17.8 mV for the adenosine-treated membranes. The effects of adenosine were determined in the presence of 30 μM dipyridamole, an adenosine transport inhihitor in neurons [25], and in the presence of 60 nM deoxycoformycin, a potent adenosine deaminase inhibitor [26].

Stimulation of $TPMP^+$ influx by 2-Cl-Ado was also observed when potassium succinate-loaded vesicles were incubated in a NaCl-containing medium (Fig. 7A). The incubation conditions employed in these experiments were identical to those used in the study

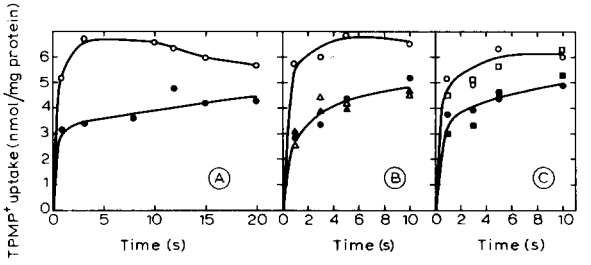

Fig. 7. The effect of 2-Cl-Ado on $TPMP^+$ uptake under control conditions (A), in the presence of theophylline (B) and in the presence of SITS (C). Potassium succinate-loaded vesicles were incubated for brief periods of time in a medium containing 56 mM NaCl and 72 mM KCl, and incubations were terminated with 0.4 M choline chloride/10 mM Tris-HCl. (A) $TPMP^+$ uptake in control samples (•) and in samples preincubated with 25 nM 2-Cl-Ado (○). Each point is the mean of triplicate determinations from a single membrane preparation with S.E. less than 10% of the mean values. Panel (B) shows the uptake of $TPMP^+$ in controls (•) and in samples preincubated with 25 nM 2-Cl-Ado (○), with 25 μM theophylline (▲) or with 25 nM 2-Cl-Ado plus 25 μM theophylline (△). Each point is the mean of triplicate determinations from a single membrane preparation with S.E. less than 15% of the mean values. Panel (C) shows the $TPMP^+$ accumulation in controls (•) and in samples preincubated with 25 nM 2-Cl-Ado (○), with 100 μM SITS (■), or with 25 nM 2-Cl-Ado plus 100 μM SITS (□). Each point is the mean of 4–6 determinations from two membrane preparations with S.E. less than 10% of the mean values.

of gramicidin's action on $TPMP^+$ accumulation (Fig. 4). These assay conditions seemed to stabilize the levels of $TPMP^+$ which were accumulated by the control and 2-Cl-Ado-treated membranes. Maximal stimulation of $TPMP^+$ influx by 25 nM 2-Cl-Ado was achieved within 3–5 s after transfer of these membrane vesicles into the NaCl-containing medium (Fig. 7). The average values of $\Delta\psi$ from four membrane preparations obtained after 5 s of incubation in this medium were: $\Delta\psi$ = −20.9 mV for control membranes and −32.9 mV for 2-Cl-Ado membranes (Fig. 7).

The methylxanthines theophylline and caffeine are known to inhibit both the electrophysiological and the biochemical actions of 2-Cl-Ado, presumably through inhibition of 2-Cl-Ado interaction with the adenosine receptor sites [1,2,21,22]. Preexposure of the synaptic plasma membrane vesicles to theophylline (25 μM) brought about nearly complete inhibition of the 2-Cl-Ado stimulation of $TPMP^+$ accumulation while it had no significant effect on basal $TPMP^+$ influx (Fig. 7B). It would appear, then, that the pharmacologic characteristics of the receptor sites with which 2-Cl-Ado interacts to produce its effects on membrane $\Delta\psi$ are quite similar to those of the sites with which this agent interacts to cause both neuronal hyperpolarization and stimulation of neuronal membrane adenylate cyclases.

Neither the 2-Cl-Ado activation of $TPMP^+$ influx nor the basal $TPMP^+$ accumulation were affected by pretreatment of the membranes with 100 μM SITS, a potent anion transport blocker in several tissues [27, 28] including neurons [29,30] (Fig. 7C). This observation suggests that the 2-Cl-Ado-induced increase in $TPMP^+$ accumulation was not produced through activation of anion transport processes which are sensitive to inhibition by SITS. Since the rapid effects of 2-Cl-Ado and adenosine on synaptic membrane $\Delta\psi$ were also clearly detectable when minimally permeant anions such as succinate were used in place of Cl^- (Fig. 5), it appears that 2-Cl-Ado increased the membrane $\Delta\psi$ primarily through enhancement of K^+ diffusion. This mechanism of action of the adenine nuceosides is quite similar to the demonstrated increase in ^{42}K efflux from taenia coli muscle cells [31] and from astrocytes in tissue culture [32], and to the consequent hyperpolarization of the muscle cells brought about by adenine nucleotides [31]. Final confirma-

tion of this proposed mechanism of adenosine action in brain synaptic membranes will, of course, require the direct measurement of the rate of either ^{42}K or ^{86}Rb efflux from the membrane vesicles.

The observations described in this report indicate that adenosine's initial action in the process of modulating the release of neurotransmitters may be to hyperpolarize the presynaptic membrane. It is quite possible that a consequence of this altered membrane polarity could be a decrease in the amount of Ca^{2+} that enters the nerve terminals with subsequent depolarizing stimuli. The results of these experiments offer indirect evidence in support of a mechanism by which adenosine could influence neurotransmitter release without the need to invoke a direct effect of the nucleoside on the voltage-sensitive Ca^{2+} channels in synaptic membranes.

Acknowledgements

This work was supported by grants from the American Heart Association, Kansas Affiliate (KSA-80-28) and from the National Institutes of Health (NS 16364). The authors wish to express appreciation to Tom Mooney, Theresa Kitos, Megan Adamson and Lorraine Hammer for their excellent technical assistance, to Dr. Hsuan Chang for her very helpful discussions, and to Jane Buttenhoff for preparation of the manuscript.

References

1 Phillis, J.W. and Edstrom, J.P. (1976) Life Sci. 19, 1 041–1 054
2 Phillis, J.W., Edstrom, J.P., Kostopoulos, G.K. and Kirkpatrick, J.R. (1979) Can. J. Physiol. Pharmacol. 57, 1 289–1 312
3 Stone, T.W. and Taylor, D.A. (1977) J. Physiol. Lond. 266, 523–543
4 Ginsborg, B.L. and Hirst, G.D.S. (1972) J. Physiol. Lond. 224, 629–645
5 Satchell, D.G. and Maguire, M.H. (1975) J. Pharmacol. Exp. Ther. 195, 540–548
6 Israel, M. and Meunier, F. M. (1978) J. Physiol. Paris 74, 485–490
7 Hedqvist, P. and Fredholm, B.B. (1976) Naunyn-Schmeideberg's Arch. Pharmacol. 293, 217–223
8 Sawynok, J. and Jhamandas, K.H. (1976) J. Pharmacol. Exp. Ther. 197, 379–390
9 Clarke, D.A., Davoll, J., Philips, F.S. and Brown, G.B. (1952) J. Pharmacol. Ecp. Ther. 106, 291–302
10 Harms, H.H., Wardeh, G. and Mulder, A.H. (1979) Neuropharmacol. 18, 577–580
11 Michaelis, M.L., Michaelis, E.K. and Myers, S.L. (1979) Life Sci. 24, 2 083–2 092
12 Fredholm, B.B. and Hedqvist, P. (1980) Biochem. Pharmacol. 29, 1 635–1 643
13 Blaustein, M.P. and Goldring, J.M. (1975) J. Physiol. Lond. 247, 589–615
14 Ramos, S., Grollman, E.F., Lazo, P.S., Dyer, S.A., Habig, W.H., Hardegree, M.C., Kaback, H.R. and Kohn, L.D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4 783–4 787
15 Cambell, C.W.B. (1976) Brain Res. 101, 594–599
16 Kanner, B.I. (1978) Biochemistry 17, 1 207–1 211
17 Chang, H.H. and Michaelis, E.K. (1980) J. Biol. Chem. 255, 2 411–2 417
18 Michaelis, M.L. and Michaelis, E.K. (1981) Life Sci. 28, 37–45
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Ramos, S., Schuldiner, S. and Kaback, H.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1 892–1 896
21 Premont, J., Tassin, J.-P., Blanc, G. and Bockaert, J. (1979) in Physiological Functions of Adenosine and Adenine Nucleotides (Baer, H.P. and Drummond, G.I., eds.), pp. 249–257, Raven Press, New York
22 Blume, A.J. and Foster, C.J. (1976) J. Neurochem. 26, 305–311
23 Williams, M. and Risley, E.A. (1980) Eur. J. Pharmacol. 64, 369–370
24 Wu, P.H., Phillis, J.W., Balls, K. and Rinaldi, B. (1980) Can. J. Physiol. Pharmacol. 58, 576–579
25 Huang, M. and Daly, J.W. (1974) Life Sci. 14, 489–503
26 Agrawal, R.P., Spector, T. and Parks, R.E., Jr. (1977) Biochem. Pharmacol. 26, 359–367
27 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
28 Barzilay, M. and Cabantchik, Z.I. (1979) Membrane Biol. 2, 297–322
29 Russell, J.M. (1980) in Anion and Proton Transport (Brodsky, W.A., ed.), pp. 510–523, New York Academy of Sciences, New York
30 Miller, C. and White, M.M. (1980) in Anion and Proton Transport (Brodsky, W.A., ed.), pp. 534–551, New York Academy of Sciences, New York
31 Jager, L.P. (1979) in Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides (Baer, H.P. and Drummond, G.I., eds.), pp. 369–376, Raven Press, New York
32 Trams, E.G. (1974) Nature 252, 480–482