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Neuronal transmembrane chloride electrochemical gradient: A key player in GABA$_A$ receptor activation physiological effect

Review Article

A. Cupello

Istituto di Bioimmagini e Fisiologia Molecolare, C.N.R., Sezione di Genova, Genova, Italy

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Summary. It has long been accepted that GABA is the main inhibitory neurotransmitter in the mammalian brain, acting via GABA$_A$ or GABA$_B$ receptors. However, new evidences have shown that it may work as an excitatory transmitter, especially in the brain of newly-born animals and acting via GABA$_A$ receptors. The difference in the end results of GABA$_A$ receptors activation in the two cases is not due to the receptor associated channels, which in both cases are chloride channels. The different physiological effect in the two cases is due to different electrochemical gradients for chloride. When GABA acting via GABA$_A$ receptors is inhibitory, either there is no transmembrane electrochemical gradient for chloride or there is one forcing such negative ions into the nerve cell, once chloride channels are open. Viceversa, GABA is excitatory when the electrochemical gradient is such to make chloride ions flow outside the cell, upon opening of the GABA activated chloride channels.

In this review this concept is discussed in details and evidence in the scientific literature for the existence of different types of chloride pumps (either internalizing or extruding chloride) is compiled.

Key words: Chloride ion – Electrochemical gradient – Neurone – GABA$_A$ receptors – Physiological effect

1 A brief history of the studies about the neurotransmitter GABA and its GABA$_A$ receptor

The first demonstrations of the presence of high amounts of γ-amino butyric acid (GABA) in the mammalian brain date back to 1950 (Awapara et al., 1950, Roberts and Frankel, 1950). The first suggestions that GABA was the main inhibitory neurotransmitter in the brain appeared in the 1950's after it was demonstrated that the inhibitory Factor I was indeed GABA (Elliott and Florey, 1956; Bazemore et al., 1957). Definite indications about its role came out from the work of Ito group in Japan about inhibition of the vestibular Deiters' neurons (Obata et al., 1967) and from the work of Krnjevic and Schwartz (1966, 1967) about inhibiton of cortical nerve cells. An important indication about the mechanism of GABA mediated inhibition in the cat cerebral cortex was that mainly chloride ions were involved (Krnjevic and Schwartz, 1967). One may say now that "ante litteram" GABA$_A$ receptors were involved (Olsen et al., 1984). By 1974 it appeared well established the concept that in the brain the main inhibitory neurotransmitter was GABA (Krnjevic, 1974). Umpteen experiments and papers have then dealt with the various aspects of GABA interaction with its receptor and physiological activity in the various brain neuronal systems. One can start mentioning binding studies of labelled GABA or agonists with brain membranes (Zukin et al., 1974; Olsen et al., 1981; and for a review: Olsen et al., 1984). Of course, of great consequence have been electrophysiological studies about the mechanisms of GABA mediated neuronal inhibition in several parts of the brain (Ayala et al., 1973; Dichter and Ayala, 1987; Miles and Wong, 1987; Mc Cormick, 1989; Edwards et al., 1990; Bernander et al., 1991; Von Krosigk et al., 1993; Thompson, 1994; Cobb et al., 1995; Whittington et al., 1995; Brickley et al., 1996; Miles et al., 1996; Hausser and Clark, 1997). The GABA receptor associated with a chloride channel has been called "GABA$_A$" starting from the description of the baclofen-sensitive and bicuculline-insensitive type of GABA receptor called “$GABA_B$” (Bowery et al., 1980; Hill and Bowery, 1981). This last type of GABA receptor is not associated with a chloride channel. A precise and inspiring synthesis of the indications gathered from the binding experiments about the $GABA_A$ receptors and the effects of modulating drugs, such as benzodiazepines and barbiturates, was published by Olsen in 1981. In this paper the $GABA_A$ receptor was rightly described as an ensemble of interacting sites: the GABA recognition site proper, the regulated chloride channel associated, the facilitatory (in case of benzodiazepine agonists) benzodiazepine site and the site(s) where picrotoxin/barbiturates (generally with opposing actions) were active.

On the other hand, starting from the beginning of the 1980’s, biochemical approaches to the purification of GABA receptors were in progress (Chang and Barnard, 1982; Martini et al., 1982; Stephenson and Olsen, 1982; Sigel and Barnard, 1984). This important research endeavour resulted in the end in an exciting turn of the story with the cloning and sequencing of the $GABA_A$ receptor α and β subunits by the Barnard group (Schofield et al., 1987, Barnard et al., 1987). The current ideas about the structure and function of $GABA_A$ receptors are summarized in the following.

According to the most recent data about $GABA_A$ receptor, it is a structure made up of five polypeptide subunits (Nayem et al., 1994) which form a chloride channel which is activated by GABA interaction with its recognition site. In the mammalian nervous system, the five subunits are from a repertoire of several classes ($\alpha,\beta,\gamma,\delta,\varepsilon,\pi,\theta$), in turn some of these classes are made up of different variants ($\alpha_{1-6},\beta_{1-3},\gamma_{1-3}$). The complexity is increased if one takes into account the possibility of splice variants, such as that which differentiates between γ_{2S} and γ_{2L}. In addition, there are three ρ subunits which do not combine with the other 17 subunits quoted and form a separate set of chloride channel associated GABA receptors, with a quite different pharmacology. This set is referred to as either “$GABA_C$” receptors (Bormann and Feigenspan, 1995; Bormann, 2000) or, according to another suggestion, is considered a specialized set of the $GABA_A$ receptors (Barnard et al., 1998). Taking into account the 17 subunits variants which can combine, one can come up with $[(17^5 - 17)/5] + 17 = 283{,}985$ possible different combinations. This calculation was originally performed by Burt and Kamatchi (1991) and gave as a result 151,887 combinations, since it was based on 15 different subunits types. Fortunately for the researchers in this field, not all combinations exist in nature. In fact, some combinations are favoured such as $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$ (Benke et al., 1994). In particular, in the cerebellum, there is a preponderance of the combinations $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2$, $\alpha_1\alpha_6\beta_{2/3}\gamma_2$ and $\alpha_6\beta_{2/3}\delta$ (Nusser et al., 1998; Zhu et al., 1998; Cupello and Robello, 2000). The subunit combinations determine the physiological characteristics of the receptors (Verdoon et al., 1990; Sigel et al., 1990) and their pharmacology (Doble and Martin, 1992; Luddens et al., 1995; Sieghart, 1995). In additon, the expression of certain subunit combinations in specific neurons of critical brain areas ultimately determines the pharmacological effect of active drugs (Rudolph et al., 1999; Mc Kernan et al., 2000; Low et al., 2000).

2 GABA is not only inhibitory via $GABA_A$ receptors

Untill the beginning of the 1990’s the general consensus was that GABA was invariably inhibitory in its synaptic action. However, it was already well established that its inhibitory activity was not always due to membrane potential hyperpolarization. In fact, it had been shown that it could directly “shunt” excitatory influences by just increasing chloride conductance in nearby membrane areas (Staley and Mody, 1992). This “shunting” effect does not necessarily involve membrane hyperpolarization. In fact, it could be operant also in conditions of depolarization of the membrane electrical potential (Staley and Mody, 1992).

However, in this case, probably restricted to dendritic domains and involving moderate depolarizations, GABA maintains an inhibitory role. When $GABA_A$ receptor mediated depolarization reaches firing threshold one must of course speak of an excitatory action by this neurotransmitter. This is what happens in postnatal (P0-P7) rat hippocampus in CA3 neurons (Ben-Ari et al., 1989; Cherubini et al., 1990, 1991; Ben-Ari et al., 1997; Leinekugel et al., 1999, 2002).

This depolarizing excitatory action of GABA via $GABA_A$ receptors either in embryonic or in immediately postnatal neurons and neuronal precursors is well established in different neuronal types and brain regions (Yuste and Katz, 1991; Reichling et al., 1994; Leinekugel et al., 1995; Lo Turco et al., 1995; Chen et al., 1996; Owens et al., 1996; Liu et al., 1997;

Owens et al., 1999; Ganguly et al., 2001). There is also indication that also the other prototypical inhibitory receptor, the glycine receptor, has excitatory actions in embryonic/neonatal neurons (Reichling et al., 1994; Sorimachi et al., 1997; Boehm et al., 1997). A relatively straightforward explanation of this phenomenon is an absence or a hypofunction of chloride extrusion mechanisms in embryonic and neonatal nerve cells (Ben-Ari et al., 1991; Rivera et al., 1999; Ganguly et al., 2001). As it will be discussed at length below, whereas a chloride extrusion mechanism causes an inward directed electrochemical gradient for chloride ions, the opposite happens when a chloride internalization mechanism is operant. In the first case, upon opening of chloride channels, there is a hyperpolarization of the neurons involved due to chloride ions inflow, the opposite happens in the second case (Misgeld et al., 1986).

$GABA_A$ receptors mediated depolarization and possibly excitation has been described in a few instances also in neurons or subcellular compartments of neurons from adult animals (Andersen et al., 1980; Alger and Nicoll, 1982; Misgeld et al., 1986; Staley et al., 1995; Kaila et al., 1997; Taira et al., 1997; Autere et al., 1999; Staley and Proctor, 1999). Misgeld et al. (1986) suggested that the depolarizing action of GABA at $GABA_A$ receptors in dendritic areas of hippocampal pyramidal neurons in adult rats was solely due to an inward directed pumping mechanism for chloride ions in such subcellular compartments. However, the common denominator of all the other instances quoted is the presence of strong stimulations, such as the application of a high frequency tetanus or the exogenous application of high GABA concentrations or strong $GABA_A$ receptors activators/modulators, such as barbiturates. In these instances however the GABA mediated depolarizations have a slow time-course and follow a first phase of rapid hyperpolarization (Thalmann et al., 1981; Staley et al., 1995; Perkins and Wong, 1996; Kaila et al., 1997; Staley and Proctor, 1999; Perkins, 1999). This phenomenon has been differently interpreted by different authors, although a common feature in these interpretations has always been the idea of outwards movements of HCO_3^- ions in the depolarization phase. One interpretation is that the rapid and conspicuous influx of chloride via activated $GABA_A$ receptors results in an increased intraneuronal chloride concentration and thus in a reduced drive for chloride influx. Under such a condition the HCO_3^- outflow prevails due to its intact driving force. In fact, as long as the extra- and intracellular pH does not change and carbonic anhydrase is active, the equilibrium potential for HCO_3^- remains not far from 0 mV (Staley et al., 1995, Staley and Proctor, 1999). Another interpretation is that in the prolonged $GABA_A$ receptors mediated depolarization phase there is activation of $GABA_A$ receptors associated to anionic channels with unusually high permeability to HCO_3^- ions (Perkins and Wong, 1997; Perkins, 1999).

The overall concept which emerges is that the chloride electrochemical gradient determines the "rapid" physiological activity of GABA via $GABA_A$ receptors. This is excitatory in the embryonic and immediately postnatal animal (Ben Ari et al., 1989; Cherubini et al., 1991), due to an electrochemical gradient which results in chloride efflux. Instead, in the adult animal it is mostly inhibitory, as amply demonstrated over the last 35 years (Obata et al., 1967; Krnjevic and Schwartz, 1967; Krnjevic, 1974; Mc Cormick, 1989; Staley, 1994; Mody et al., 1994; Low et al., 2000). However, in the adult animal under conditions of strong stimulation the $GABA_A$ receptors mediated signal may become depolarizing and excitatory due to the coming into play of the HCO_3^- ions. This signal has a more prolonged time course in the respect of those mediated solely by chloride ions. From a physiological point of view, this condition may correspond to a strong excitation which adds to the glutamatergic one, possibly resulting in plastic changes in the target neuron.

A still different instance is when neurons undergo trauma resulting in a change of the electrochemical gradient for chloride (Van den Pol et al., 1996; Van den Pol, 1997). A possible related phenomenon is the emergence of depolarizing $GABA_A$ mediated signals after ischemic-like insults in neurons (Fukuda et al., 1998). These latter instances possibly represent cases in which the chloride pump activity of the neurons changes, as a consequence of a different expression of mechanisms of active transport of chloride. We will discuss at length below the different types of chloride pumps so far described. Another point in this context is that the passage of the chloride mediated $GABA_A$ signal from depolarizing/excitatory to hyperpolarizing/inhibitory during development is mediated by switches in the expression of chloride pumping mechanisms, from internalizing to extruding ones. This process may be mediated by GABA itself as a neurotrophic agent (Ganguly et al., 2001).

3 Transmembrane electrochemical gradients of significance for GABA physiological activity mediated by $GABA_A$ receptors

The Goldman-Hodgkin-Katz equation describes the nerve cell membrane potential under steady-state condition as a function of the main intra- and extracellular monovalent ionic species and their permeability:

$$
\begin{aligned}
E = \ & RT/F \ln[(\Sigma_l p_l \times [C_l^+]_e \\
& + \Sigma_t p_t \times [A_t^-]_i)/(\Sigma_l p_l \times [C_l^+]_i \\
& + \Sigma_t p_t \times [A_t^-]_e)]
\end{aligned}
$$

Where	$C_l{}^+$	represents the general monovalent cation
	$A_t{}^-$	represents the general monovalent anion
	p_l and p_t	represent their permeabilities across the plasma membrane.

If activation of $GABA_A$ receptors opens anionic channels with a $pCl^- : pHCO_3^- = 5:1$ (Bormann et al., 1987):

$$
\begin{aligned}
E_{eq\,GABA} = \ & RT/F \ln[([Cl^-]_i + 0.2 \times [HCO_3^-]_i)/([Cl^-]_e \\
& + 0.2 \times [HCO_3^-]_e)]
\end{aligned}
$$

Because all the others p's become insignificant. At physiological extra- and intracellular pH of respectively 7.4 and 7.2, the extra- and intracellular concentrations of bicarbonate ions can be evaluated as respectively 26 and 16 mM (Staley et al., 1995). Thus:

$$E_{eq\,GABA} = RT/F \ln[([Cl^-]_i + 3.2)/([Cl^-]_e + 5.2)].$$

Taking into account that average values for $[Cl^-]_i$ and $[Cl^-]_e$ are respectively 6 and 120 mM, one realizes that relatively small changes in $[Cl^-]_e$, of the order of few mM, cannot influence much $E_{eq\ GABA}$. The opposite is true for for small changes in $[Cl^-]_i$.

However, in the presence of an inward directed chloride pump (such as in neurons in the early development, see paragraph above) $[Cl^-]_i$ may be much higher than 6 mM. For instance if the $E_{eq\ GABA}$ is as depolarized as −40 mV, $[Cl^-]_i$ may be as high as 24 mM. Thus, under these circumstances the 3.2 term contributed by HCO_3^- becomes almost negligible. This explains why the HCO_3^- contribution to $GABA_A$ receptor current is not in play when this current is decidedly inward such as in early postnatal neurons (Ben Ari et al., 1991).

Viceversa, under circumstances in which the $GABA_A$ current is conspicuously outward, such as in the experiments by Staley and Proctor (1999), there the starting $[Cl^-]_i$ is lower than 6 mM. Thus, such $[Cl^-]_i$ is of the order of magnitude of the contribution by HCO_3^-. Under these circumstances the HCO_3^- contribution may become significant (Staley et al., 1995; Staley and Proctor, 1999).

4 Early electrophysiological studies about IPSP's in neurons suggesting the existence of chloride pumps

Differences between chloride equilibrium potential (E_{Cl}) and the resting membrane potential (E_m) in either neurons or muscles have been described since many years (Mauro, 1954; Boistel and Fatt, 1958; Keynes, 1963; Neild and Thomas, 1974; Ascher et al., 1976). In these cases the existence of chloride pumps has been postulated (Boistel and Fatt, 1958; Keynes, 1963). These suggestions are born out by experimental results in many neuronal systems. In particular, in the giant neuron of the *Aplysia* abdominal ganglion there is an active outward transport of chloride which is reversibly suppressed by cooling (Russel and Brown, 1972). The same applies to cat trochlear motoneurons (Llinas et al., 1974), to cat spinal motoneurons (Lux et al., 1970; Lux, 1971; Iles and Jack, 1980), cortical neurons (Raabe and Gumnit, 1975) and frog spinal cord motoneurons (Nicoll, 1978) where the process is reversibly blocked by ammonium ions. A similar circumstance applies also to stretch receptor neurons in the crayfish (Meyer and Lux, 1974). An analogous effect may have furosemide (Deisz and Lux, 1976), although according to another author the furosemide effect might be due to a direct blockade of chloride channels (Nicoll, 1978). Failures of ammonium ions to abolish hyperpolarizing IPSP's in hippocampal pyramidal cells have been reported (Allen et al., 1977; Alger and Nicoll, 1983). An ammonium ion inhibited inward chloride transport has been proposed by Buhrle and Sonnhof (1985) to explain depolarizing IPSP's in frog spinal cord motoneurons.

Further studies have revealed a rather complicated situation in the guinea pig hippocampal CA3 region, where pyramidal neurons appear to exhibit hyperpolarizing cell body IPSP's and depolarizing IPSP's in dendrites (Misgeld et al., 1986). A similar circumstance had been described previously in rat CA1 pyramidal neurons (Andersen et al., 1980; Thalmann et al., 1981). In the experiments by Misgeld et al., also dentate gyrus granule cells showed depolarizing IPSP's. These authors postulated the existence of two different chloride pumping mechanisms on the very same CA3 pyramidal cells: one on the cell body which extrudes chloride and another one on dendrites internalizing it. Thus, in the cell body E_{eq} of the GABA dependent IPSP is more negative than E_m, the opposite happens in the dendrites. This same circumstance applies also to dentate gyrus granule cells. Both chloride transport mechanisms were shown to be inhibited by furosemide (Misgeld et al., 1986). The outward mechanism was later suggested by the same group to be due to K^+/Cl^- outward cotransport (Muller et al., 1989). The existence of a different response to the inhibitory neurotransmitter GABA in the cell body (hyperpolarization) and in dendrites (depolarization) in adult hippocampus neurons was confirmed by Huguenard and Alger (1986) with neurons dissociated from guinea pig hippocampus. Actually, an uneven distribution of chloride ions has been described in hippocampal neurons by Hara et al. (1992). The heterogeneity in intracellular chloride concentrations obviously can be found also between different cell types, such as fast-spiking interneurons and regular-spiking principal cells in the cortex and amygdala of mature animals, being due to different chloride transporting mechanisms (extruding vs. internalizing ones; Martina et al., 2001). The existence of a K^+/Cl^- outward cotransport has been discussed as an explanation of hyperpolarizing IPSP's in adult guinea pig cortex and hippocampus (Thompson et al., 1988a, 1988b and 1989). The emergence of an outward pumping mechanism for chloride has been suggested also by Zhang et al. (1991) in order to explain the passage from non-hyperpolarizing to hyperpolarizing IPSP's in rat CA1 pyramidal neurons during early postnatal development. In this context it seems also interesting to recall the demonstration of the existence on the membrane of rat hippocampus CA1 pyramidal neurons of a voltage dependent chloride conductance ($G_{Cl(V)}$) with strong inward rectification allowing the passage of chloride ions only in the in→out direction, if the chloride electrochemical gradient commands that. If the electrochemical gradient is the opposite, it does not allow any chloride permeation (Staley, 1994). Staley suggested that its differential expression in the postnatal developmental period may contribute to the switch of the GABA mediated IPSP's from depolarizing to hyperpolarizing (Cherubini et al., 1990; Zhang et al., 1991). Its down-regulation is a necessary condition for the establishment of excitatory GABA activities, implying chloride outflows, which otherwise would be occluded.

5 Nature and stoichiometry of chloride active transport mechanisms

The papers by Thompson et al. suggesting the existence in mammalian cortical/hippocampal nerve cells of a K^+/Cl^- cotransport have been mentioned above (Thompson et al., 1988a, 1988b, 1989). This mechanism appears to be suitable to produce an inward directed chloride gradient leading to hyperpolarizing IPSP's. In fact, the process $K^+_i + Cl^-_i \leftrightarrow K^+_e + Cl^-_e$ has:
$\Delta G = RT/F \ln([K^+]_e \times [Cl^-]_e / [K^+]_i \times [Cl^-]_i)$, since at equilibrium $\Delta G = 0$ it derives that at equilibrium $[K^+]_e \times [Cl^-]_e = [K^+]_i \times [Cl^-]_i$. This corresponds to the Donnan equilibrium across the plasma membrane for the two ions. On the other hand, the potassium potential $E_K{}^+$ is:
$RT/F \ln([K^+]_e/[K^+]_i)$. Thus, the $E_{Cl^-} = E_{IPSP} = RT/F \ln([Cl^-]_i/[Cl^-]_e) = RT/F \ln([K^+]_e/[K^+]_i) = E_K{}^+$. Taking into account that $E_K{}^+ \cong -100\,mV$, it comes out that in these cases the IPSP's are strongly hyperpolarizing. Four K^+/Cl^- cotransporters have been molecularly characterized: KCC1 (Gillen et al., 1996), KCC2 (Payne et al., 1996) and KCC3, 4 (Mount et al., 1999). The first one is present in glial cells whereas the second one is neuronal and present in neurons from several parts of the brain (Payne et al., 1996; Gulyas et al., 2001). The functional characteristics of KCC2 have been characterized by transfection in HEK-293 cells (Payne, 1997). Also KCC3 is present in neurons (Pearson et al., 2001).

In rat hippocampus pyramidal neurons the switch of $GABA_A$ receptors mediated response from depolarizing to hyperpolarizing in the early postnatal period is accompanied by the expression of KCC2 (Rivera et al., 1999). An analogous situation appears to be present in lateral superior olive neurons in rats, where the early postnatal development is accompanied by the emergence of a chloride extruding K^+/Cl^- cotransporter, whereas a $Na^+/K^+/2Cl^-$ transporter appears to be present already in immature neurons (Kakazu et al., 1999). In this case the "inhibitory" neurotransmitter involved is glycine. The existence of a K^+/Cl^- cotransporter has also been demonstrated in rat midbrain neurons (Jarolimek et al., 1999). The development of antibodies against the cotransporter KCC2 (Williams et al., 1999) has allowed the immunohistochemical study of its developmental appearance in various parts of the rat retina (Vu et al., 2000). Experiments by De Fazio et al. (2000) confirmed a low presence of KCC2 in rat neocortical pyramidal neurons from immature animals. In addition, these experiments showed that in cortical neurons from mature animals KCC2 activity could result in either accumulation or depletion of intracellular chloride according to the extracellular K^+ concentration. Evidently, the evaluation made above of the consequences of K/Cl cotransporter activity on E_{Cl} (and E_{IPSP}) applies to "normal" extracellular concentrations of K^+. In hippocampal neurons cultures from rat embryos, it has been shown that an inactive form of KCC2 can be activated during subsequent maturation of the neurons, possibly by protein tyrosine phosphorylation (Kelsch et al., 2001). In any case, a higher expression of KCC2 in neurons is correlated with a more efficient extrusion of chloride and a better maintainance of hyperpolarizing IPSP's in relatively mature animals (Ueno et al., 2002). Also KCC3 is expressed in the brain and spinal cord and it presents an increase during postnatal development (Pearson et al., 2001). Another candidate as a possible chloride extrusion mechanism is a Cl^- – ATPase, which has been described by Shiroya et al. (1989) and shown by this group to extrude chloride from rat hippocampal neurons (Inoue et al., 1991; Hara et al., 1992). Activity of a Cl^- – ATPase is also implied in studies about hyperpolarizing actions of GABA in CA3 neurons (Bonnet et al., 1996).

A final comment within the context of the developmental maturation of the extrusion systems for chloride is that, despite all the evidence discussed above, KCC2 appears of importance in the rodent already at the early postnatal period. In fact, KCC2 knockout mice die immediately after birth because of severe motor deficit and lack of respiration (Hubner et al., 2001).

Referring to the active accumulation of chloride which must be at the basis of depolarizing IPSP's a likely candidate is a $Na^+/K^+/Cl^-$ cotransporter, which has already been described to mediate chloride influx into the squid giant axon (Russel, 1983). This mechanism has been suggested also for intracellular chloride accumulation into mammalian neurons (Hara et al., 1992; Rohrbough and Spitzer, 1996; Takebayashi et al., 1996). A suggested stoichiometry for this electroneutral process is $Na^+/K^+/2Cl^-$ (Alvarez-Leefmans et al., 1988). At equilibrium, the ΔG associated with this process must be zero, thus: $RT/F \ln([Na^+]_i \times [K^+]_i \times [Cl^-]_i^2/ [Na^+]_e \times [K^+]_e \times [Cl^-]_e^2) = 0$; from this it comes: $[Na^+]_i \times [K^+]_i \times [Cl^-]_i^2/ [Na^+]_e \times [K^+]_e \times [Cl^-]_e^2 = 1$. In other words, at the equilibrium:
$([Cl^-]_i/[Cl^-]_e)^2 = ([Na^+]_e/[Na^+]_i) \times ([K^+]_e/[K^+]_i)$ or $2 \times RT/F \ln([Cl^-]_i/[Cl^-]_e) = RT/F \ln([Na^+]_e/[Na^+]_i) + RT/F \ln([K^+]_e/[K^+]_i)$.
Taking into account the most frequent equilibrium potentials of Na^+ and K^+ in neurons as respectively $+40\,mV$ and $-105\,mV$ it comes out $-32.5\,mV$ as the equilibrium potential of Cl^-. This value is very close to the $-35\,mV$ evaluated as the E_{Cl^-} in hippocampal neurons dendrites by Hara et al. (1992).

Interestingly, the expression of the BSC2 isoform of the Na-K-2Cl cotransporter (Delpire et al., 1994) has been shown to be developmentally regulated in the postnatal rat brain, with a high presence in the cerebral cortex and hippocampus in the first postnatal week and a lower level from postnatal day (PND) 14 to adulthood. The signal in the cerebrum and cerebellum white matter peaked at PND 14 (Plotkin et al., 1997). This result is of obvious interest since it nicely explains the known depolarizing-excitatory effect of $GABA_A$ receptor activation in hippocampal neurons only in the first postnatal week (Ben-Ari et al., 1989; Cherubini et al., 1990). In fact, in the presence of this cotransporter, chloride would be enriched intracellularly above its Nernst equilibrium at the resting E_m. Thus, under the action of GABA at $GABA_A$ receptors it would tend to exit from the cells and depolarize them. A Na/K/2Cl cotransporter was demonstrated also in immature rat cortical neurons (Sun et al., 1999). The molecular mechanisms of NaCl cotransport in the kidney has been reviewed in a relatively recent paper (Kaplan et al., 1996).

Dorsal root ganglion neurons from mice null for the Na/K/2Cl cotransporter NKCC1 (Delpire et al., 1999) showed no GABA activated chloride inward currents at variance with the wild type animals, indicating the absence of active chloride intracellular accumulation (Sung et al., 2000). NKCC is also probably involved in the generation of an outward directed chloride electrochemical gradient in glutamatergic nerve terminals on ventromedial hypothalamic neurons, where GABA presynaptically stimulates glutamate release via GABA$_A$ receptors (Jang et al., 2001).

6 A neuronal chloride extrusion mechanism based on a weird GABA$_A$ receptor type

A different chloride extrusion mechanism has been proposed by our group in Genova together with Holger Hydén (Goteborg). This suggestion derives from a long series of experiments based on the use of single nerve membranes microdissected from adult rabbit vestibular Deiters' neurons (Hydén et al., 1980). These single membranes can be studied in a microchamber system from the point of view of labelled chloride permeation in the two opposite directions out↔in across the membrane (Hydén et al., 1986) and of the effect of GABA. These experiments have shown that GABA on the intracellular side of these membranes can stimulate chloride in→out permeation with a pharmacology analogous to that of GABA$_A$ receptors (Hydén et al., 1999). This effect presents an abrupt desensitization at intracellular GABA concentrations $\geq 10^{-5}$M. Another characteristic is that these "GABA$_A$ receptors", with the GABA recognition sites on the membrane inside, expose positive charges at the intracellular mouth of the associated chloride channel (Hydén et al., 1999). These characteristics have suggested us that these "receptors" are GABA activated chloride extrusion pumps, where the energy for chloride extrusion is provided by ATP in a phosphorylation step within the extrusion cycle (Rapallino et al., 1993; Hydén et al., 1999, 2000). In particular, we suggested (Rapallino et al., 1999) that the mechanism proposed was a particular instance of a possibility which had been discussed theoretically before by the german biophysicist Peter Lauger (1979, 1991). That is the possibility that an ionic channel may function as an ionic pump if energy is provided by ATP. The core mechanism is the inversion of two energy peaks along the permeation pathway. This would cause that an ion entering the channel from one (say: intracellular) side of the channel overcoming the lowest peak of energy would be facilitated, after the peak inversion, to overcome another one on the other (say: extracellular) side and exit (see Fig. 1A, redrawn from a figure of Lauger, 1991). The end result is the extrusion of the ion at the expense of ATP consumed in a step (phosphorylation?) leading to the inversion of the

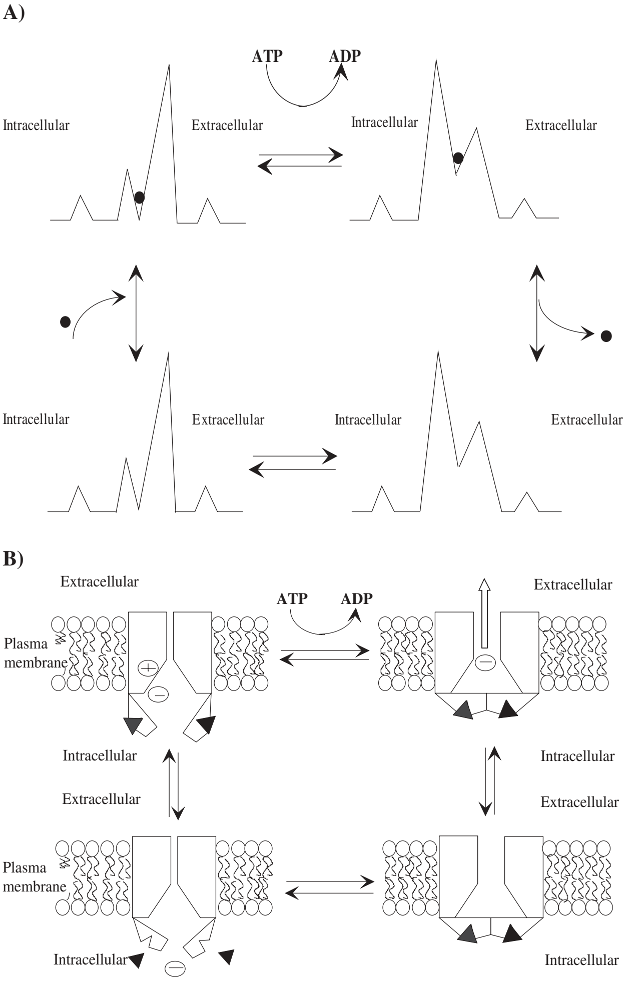

Fig. 1. **A** This scheme is modified from a previous one published by Lauger (1979, 1991; see reference list) and shows how an ion (represented as a black ball) can be extruded (in → out) from a cell via an ionic channel if energy is provided to the system by ATP consumption. The scheme represents the energy peaks and wells met by the ion along its permeation pathway. A central energy well is initially reached by the ion from the left (intracellular side) because the lowest energy peak is on the left of the energy well. Then, due to phosphorylation, the lowest energy peak is on the other (extracellular) side of the well. In this way an ion is extruded at the expense of ATP. **B** A scheme parallel to the one in part **A**. However, in this case the figure involves the representation of the chloride channel which is activated by intracellular GABA molecules (black triangles). The scheme shows how a chloride ion (the white circle with a minus sign) is attracted to the channel cytoplasmic vestibule by an exposed positive charge. Then, a phosphorylation step occurs with the disappearance of the positive charge and closing of the cytoplasmic gates. The entrapped anion has the possibility of escaping the highly electrically charged enclave only by passing to the cell outside. Finally, the system can go to the initial configuration and begins a new cycle

height of the two energy peaks along the permeation pathway. In the case of the process suggested by us the core steps involved are illustrated in Fig. 1B.

We suggest that this chloride extrusion process is present in the vestibular Deiters' neurons. An important difference with the K^+/Cl^- cotransporter is that the latter is electrically neutral whereas the former is actually electrogenic. An intracellular GABA activated mechanism of chloride extrusion would be particularly suitable for a neurone which accumulates GABA postsynaptically, such as the Deiters' cell (Storm-Mathisen, 1975; Okada and Shimada, 1976).

7 Conclusions

GABA may exert an inhibitory effect on neurons via $GABA_A$ receptors activation without any change of membrane potential. This can be achieved by just increasing membrane conductance to chloride and "shunting" the excitatory inputs. However, inhibition is even more efficacious if it involves membrane hyperpolarization. On the other hand, in particular cases, such as during early development of the brain, GABA can be depolarizing and excitatory. The pivotal player is the preexisting chloride electrochemical gradient. In the former case this gradient is inward and due to a chloride extrusion mechanism. In the latter case the chloride electrochemical gradient is outward and built by a chloride internalization active process. Under particular conditions and in the adult animal, also HCO_3^- ions outflow may come into play in GABA mediated excitation.

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Author's address: Dr. Aroldo Cupello, Istituto di Bioimmagini e Fisiologia Molecolare, Via De Toni 5, I-16132 Genova, Italy, Fax: 39-010354180, E-mail: dcupel@neurologia.unige.it